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DENDRITIC SPINES AND MEMORY FORMATION IN THE CHICK

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ABSTRACT

Passive avoidance training results in a number of biochemical, morphological, and electrophysiological changes in the forebrain of the one day-old chick. One particular region in which these alterations occur is the intermediate part of the medial hyperstriatum ventrale (IMHV). This thesis reports several morphological experiments on the effects of passive avoidance training on dendritic spines and dendritic branching patterns of large, multipolar, projection neurons (abbreviated LMPNs) in the IMHV, in order to determine structural correlates of memory formation for this task, at the light microscope level.

The chicks were trained on a passive avoidance training task by presentation of a shiny chrome bead coated with a bitter tasting substance (methylantranilate). The chicks will spontaneously peck at the bead, but show a characteristic disgust response on the first peck and avoid a similar (dry) bead subsequently (trained or M-chicks). Control chicks were presented with a water coated bead, which they do not find aversive (W-chicks). 24-26h later, the chicks were perfused with an aldehyde fixative, and the left and right IMHV regions were dissected out and Golgi-impregnated by the rapid Golgi method. The blocks were sectioned with a tissue chopper at 90-120 μ m, and permanently mounted onto slides, in DPX, which were coded so that subsequent procedures were performed "blind".

LMPNs in the left and right IMHV regions from trained and control chicks were identified and examined for changes in spine density, spine shape and in dendritic branching patterns (which were analysed by the Sholl concentric ring method, by the number of dendrites at each branch order and by vertex analysis).

Training resulted in (1) a significant increase in spine density in the left and right hemispheres and (2) an increase in the mean diameter of the spine heads with concomitant shortening of the spine stems, but only significantly in the left hemisphere. These changes occurred without a significant change in the mean overall spine length and also without significant alterations in the lengths or the diameters of the dendrite branches. A significant hemispheric asymmetry was also observed between the left (L) and right (R) hemispheres of control chicks: $R > L$, but no asymmetry was found in trained chicks.

No significant differences in branching patterns were found after passive avoidance training.

Because the changes in spine density and shape may have been caused by non-specific factors associated with the training experience, such as stress, arousal, or the taste of the methyl anthranilate, *per se*, a further experiment was conducted in which trained chicks were given a brief, subconvulsive, transcranial electroshock, 5 min after showing the disgust response. This rendered approximately half of the chicks amnesic, the rest showed recall when tested 24h after training. Control chicks receiving the same treatment showed no change in pecking behaviour. A significant increase in spine density was found in the recall group compared with either the amnesic or the shocked water control groups, but only in the left hemisphere. However, no alterations in spine shape and no significant differences in dendrite lengths or dendrite diameters were noted.

In conclusion: (1) passive avoidance training is associated with an increase in spine density that is specifically related to long-term memory formation for the task; (2) spine density and shape changes can occur within 24h of a single-trial learning

experience; (3) these alterations occur without significant differences in branching patterns of the LMPNs, suggesting either that it may take longer than 24h for observable changes in branching patterns to occur, or that they may not be involved in memory storage for the training task, and (4) because the shape changes were only found on LMPNs in the left hemisphere of trained chicks, this hemisphere may be predominantly involved in the memory storage processes for this task. This is also supported by qualitative results from the dendritic branching investigation.

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ABBREVIATIONS

cAMP:	Cyclic adenosine monophosphate,
CXM:	Cycloheximide,
DD:	Dendrite diameter,
DGG:	γ - D-glutamylglycine,
DL:	Dendrite length,
EPSP:	Excitatory postsynaptic potential,
GABA:	γ - aminobutyric acid,
HA:	Hyperstriatum accessorium,
IMHV:	Intermediate part of the medial hyperstriatum ventrale,
IP3:	Inositol trisphosphate.
LGN:	Lateral geniculate nucleus,
LMPN:	Large, multipolar, projection neuron,
LPO:	Lobus parolfactorius,
LTP:	Long-term potentiation,
MAPs:	Microtubule associated proteins,
n:	Number of visible spines,
N:	Estimate of the total number of spines,
NMDA:	N-methyl-D-aspartate,
PA:	Palaeostriatum augmentatum,
PKC:	Protein kinase C,
PS:	Phosphatidylserine,
RA:	Robustus archistriatalis,
SD:	Spine density,
SHD:	Spine head diameter,
STL:	Spine stem length,

Some of the results from this thesis have been published in following journals:

Patel, S. N., and Stewart, M. G. (1988). Changes in the number and structure of dendritic spines 25h after pasive avoidance training in the domestic chick, *Gallus domesticus*. *Brain Research*, 449: 34-46.

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Patel, S. N. and Stewart, M. G. (1988). Passive avoidance training causes alterations in dendritic spine parameters of large, multipolar projection neurons in the chick IMHV. *Neuroscience letters, suppl.* 32: S47.

Patel, S. N., and Stewart, M. G. (1987). Passive avoidance training causes increases in spine density in the chick IMHV. *Neuroscience letters, suppl.* 29: S100.

Copies of the major papers are attached to the back of this thesis.

CHAPTER 1

GENERAL INTRODUCTION: MEMORY

One of the most fundamental processes of the central nervous system is the processing and subsequent storing of information about the environment with which the organism constantly interacts. The newborn animal knows little about the world into which it arrives, and in order to survive must learn much about the environment, e.g. to avoid predators, and to identify the locations of food sources. Some of this vital information may be learnt by imitation while the animal is young. This information then forms the foundation upon which more and more information can be built as the animal progresses through life.

There are many definitions of learning and memory. A general, operational definition which a neurobiologist studying animal behaviour may use is that learning involves the acquisition of new patterns of behaviour in response to internally (e.g. needs for food or water) or externally (e.g. avoiding predators, or electric shock in the experimental situation) motivated events. Memory therefore constitutes the neural representations of the learnt information which outlasts the actual event and is preserved in the neuronal organisation of the brain for months or years. These internal neural mechanisms by which an organism processes, learns, stores and then recalls information has been a mystery for centuries. Ramon y Cajal (1893) suggested that the formation of new synaptic connections was itself the basis of learning and memory storage. However, Tanzi (1893) preferred the idea that a change in the strengths of previously existing connections was the mechanism responsible for engram formation. The most favourable idea today is the "consolidation" hypothesis of memory storage, first proposed by Müller and

Pilzecker in 1900. Their basic tenet was that the learnt information is retained in the nervous system for sufficient time to produce changes in the neuronal structure which results in consolidation of the experience as a neural memory trace. Hebb (1949) later elaborated upon this idea, suggesting that reverberatory activity within a given neural circuit will lead to changes in the efficacy of activated synapses, thereby storing the neural trace representing the learnt information. Re-activation of the trace will retrieve the information concerning the event.

For such structural alterations to occur there must be corresponding, transient, biochemical changes in RNA and protein synthesis which are correlated with the learning experience (review Dunn, 1980; Shashoua, 1982; Agranoff et al, 1976; Glassman, 1969; Rose and Haywood, 1976). Hydén and Egyhazi (1962) initiated the search for these neurochemical correlates of learning. They found a change in RNA metabolism in the hippocampus of rats which were trained to eat with their non-preferred forepaw. Enhanced ^{14}C -leucine incorporation into two specific brain proteins, S-100 and 14-3-2 (review: Moore, 1972) into the entorhinal and septal areas of the rat brain (Hydén and Lange, 1970a), was also reported after a hand-reversal task and in rat brain hippocampal cells after a passive avoidance conditioning paradigm (Gluschchenko et al, 1977). Hydén and Lange (1970b) further showed that these proteins may be necessary biological correspondents of memory formation by injecting antisera prepared against the S-100 protein into trained rats which subsequently became amnesic for the hand-reversal task.

Similar biochemical changes have also been found in chicks after imprinting and passive avoidance training, even though these two learning events are very different in their nature. That is, whereas imprinting involves approach behaviours towards objects which the chick finds attractive (most often which it identifies as the

"mother figure"), passive avoidance training teaches the chick to avoid a particular object (a chrome bead, in this case). There is enhanced mRNA synthesis, as a result of imprinting in chicks (Horn et al, 1973a; review: Horn, 1985) including enhanced incorporation of amino acids, such as ^3H -lysine into the forebrain roof of chicks after imprinting (Hambley et al, 1977). Passive avoidance training in one day old chicks also shows enhanced incorporation of ^3H -fucose into glycoproteins (Sukumar et al, 1981; Burgoyne and Rose, 1981; McCabe and Rose, 1984; Rose and Harding, 1984), and ^{14}C -leucine into tubulin (Mileusnic et al, 1981), which lasts for up to 24h. These and many other similar reports of changes in protein synthesis corresponding to learning a variety of tasks in a number of animals, in particular mice and rats, suggests that there may be general biochemical mechanisms underlying the processes of memory formation in different animal species and for different learning tasks [e.g. in addition to the above; ^3H -valine incorporation in a swimming task in goldfish (review: Shashoua, 1982); uridine incorporation into the hippocampus in a thirst motivated Y-maze task for rats (Bowman and Kottler, 1970); orotic acid incorporation in a dark avoidance and spatial discrimination task in rats (Hershkowitz and Samuel, 1974); ^{32}S -met and ^3H -lysine in an operant conditioning paradigm in mice (Herschkowitz et al, 1975); and ^{14}C -leucine incorporation after shock-motivated visual discrimination tasks in rats (Krivanek, 1974, 1975)].

Many other model systems have been devised in order to understand the processes governing these learning-induced changes in biochemistry and the resulting alterations in the structure of synapses underlying long-term memory storage. It is not intended here to review the results from each of these systems in this general introduction, but only to introduce the concepts governing the use of these models

as they pertain to the issue of learning and memory. The many invertebrate model systems, such as *Aplysia* (Abrams and Kandel, 1988; Pinsker et al, 1970; Abrams, 1985), *Hermissenda* (Alkon, 1974; Crow, 1988), and *Drosophila* (Quinn et al, 1979), have provided us with a better understanding of the basic neuronal mechanisms underlying processes such as sensitization, habituation, and associative learning. For example, in *Aplysia*, repeated mild stimulation of the siphon results in a decrease in gill withdrawal, which, with repeated training sessions, can be maintained for weeks (long-term habituation) (Pinsker et al, 1970; Carew et al, 1972). Long-term sensitization can be induced by applying electric shocks to the mollusc's foot or head - subsequent mild stimuli applied to the siphon will elicit unusually vigorous gill withdrawal (Carew et al, 1971). Bailey and Chen (1983) have shown that long-term habituation and sensitization of the gill-withdrawal reflex produces morphological changes in the sensory neurons. The number, size, and vesicle complement of sensory neuron active zones are larger in sensitized animals than controls, and smaller in habituated animals. They also show an increase in the number of presynaptic varicosities in sensitized animals and a decrease in habituated animals, compared to controls. Thus even so-called "basic" forms of learning in "lower" animals produces observable alterations in the morphology of identified neurons.

Another important model of neural plasticity developed in the last two decades, is long-term potentiation (LTP) (Bliss and Lomo, 1973). Brief trains of high frequency stimulation of a number of excitatory hippocampal pathways can result in potentiation of synaptic transmission lasting for hours or days (review: Bliss, 1979). The attraction of this model is that it tests the Hebb hypothesis of persistent neural activity causing long-term synaptic changes in the stimulated neural pathways (Kelso et al, 1986; DiPrisco 1984). At least in partial support for Hebb's

idea, such stimulation is known to cause changes in the structure of synapses and spines. For example, perforant path stimulation causes an increase in the numbers of shaft and sessile spine synapses (Chang and Greenough, 1984), and an increase in the volume of spines (Fifkova and van Harreveld, 1977). This model therefore allows a direct simulation of what may be occurring during learning.

Similar observations have also been made in vertebrate model systems of learning. For example, rats trained on a Y-maze to avoid a shock in a visual discrimination task show a 32% increase in the number of synapses in the CA3 region of the hippocampus compared to quiescent controls, or in pretrained animals, that were actively performing the task (Wenzel et al, 1977). These alterations occurred within 30 min of the training, but were not present in animals which had prolonged Y-maze training, and were therefore changes of a transient nature. Multiple training in a Hebb-Williams maze also shows increases in the number of synapses, as indicated by greater dendritic branching of layers IV and V pyramidal neurons of the occipital cortex (Greenough et al, 1979). Turner and Greenough (1985) have shown that light microscopic measures of postsynaptic surface changes in dendritic branching and spine number, accurately reflects electron microscopic estimates of synaptic density. They performed an electron microscopic investigation of the occipital cortex of rats reared from weaning in an enriched environment (group cages with different objects changed daily - EC rats), social environment (in group cages - SC rats), and isolated conditions (- IC rats), for thirty days, and found a significant increase in the number of synapses per neuron in the EC and SC rats over the IC rats (EC>SC). These changes are also correlated with increased dendritic branching (Volkmar and Greenough, 1972) and increased spine density (Globus et al, 1973), of pyramidal neurons in the same region in the occipital cortex

of EC rats.

There is also much evidence in the literature reporting alterations in pre-existing synapses as a result of learning (reviews: Greenough and Bailey, 1988; Greenough and Chang, 1985), although it is difficult to rule out the possibility of the formation of new synapses with different characteristics in these studies. On the assumption that neural communication occurs at the synaptic junction where the receptors for the neurotransmitters are located, an increase in the size of the synaptic contact zone, is likely to mean an increase in the number of receptors and hence an increase in efficacy of that synapse. This could occur through the postsynaptic density occupying a larger area, which would show up as an increase in its length in the electron microscope (after relevant stereological corrections have been made). Such increases have indeed been observed after imprinting in the intermediate part of the medial hyperstriatum ventrale (IMHV) region of the left hemisphere in two day old chicks (Horn et al, 1985). McCabe and Horn (1988, in press) have further shown that this may also represent an increase in the number of NMDA receptor sites in the left IMHV after imprinting. Increases in the number of muscarinic cholinergic receptors have also been found after a specific one-trial passive avoidance learning task in the chick (described below) (Aleksidze et al, 1981; Rose et al, 1980). More synaptic vesicles may also indicate a greater amount of neurotransmitters. Indeed, 24h after passive avoidance training, Stewart et al (1984) have found an increase in the numerical density of vesicles per synapse in the left IMHV region in the chick, together with an increase in the volume density of presynaptic boutons. Similar increases in the size of the synaptic contact zones have also been found in the occipital cortex of rats that have been reared from weaning in a complex environment compared to isolates or rats group housed for thirty days (Sirevaag and Greenough, 1985), but not in adult rats kept in these environments (Hwang

and Greenough, 1986).

Another important mechanism by which the efficacy of synapses can be increased is by altering the dimensions of the postsynaptic receptive surface, i.e. that of dendritic spines, which in mammalian occipital cortex neurons represent about 80-90% of the postsynaptic receptive sites. The mere presence of a synapse on a dendritic spine can affect the excitatory postsynaptic potential, which can either be attenuated or amplified, depending on the cytoplasmic resistance afforded by the spine stem and spine head. Thus, there are optimal values for the spine stem and spine head dimensions for which the EPSP can be maximally amplified (cf. "function of dendritic spines"). The fact that dendritic spines are present in a wide variety of taxa and are observed to change in both number and shape upon learning or sensory stimulation in animal models, in humans with childhood mental disorders and in adult dementias, suggests that spines must subserve some important functions, one of which may be in learning and memory processes.

Indeed the nervous systems of phylogenetically diverse species of animals have the same distinct anatomically identifiable units, 'neurons', although these units may be interconnected to form neuronal networks of varying degrees of complexity. Therefore, it seems not unreasonable to suggest that the mechanisms involved in the storage of information in organisms will have many similarities, even for different learning tasks and may also show similarities across different species. This argument is further supported by observations of similar structural changes occurring in different animal species and after different learning experiences (see below).

The aims of this project were therefore to investigate possible postsynaptic correlates of information storage for the passive avoidance training task, in particular changes in branching pattern of dendrites and in the morphology and number of dendritic spines. Most importantly, the nature of the passive avoidance training task also allowed a determination of the specific relationship between any changes in dendritic parameters in trained chicks and memory formation, because it was possible to observe any comparable changes in amnesic chicks. The passive avoidance learning task briefly involves presenting chicks with a bright chrome bead, at which they spontaneously peck. If the bead is coated with a bitter tasting substance (methyl anthranilate) the chick will peck once, show a characteristic disgust response and subsequently avoid a similar, but dry bead (M-chicks). Control chicks peck and do not avoid a water coated bead (W-chicks).

This paradigm is a good model for studying memory formation processes primarily because it is a one-trial training event in which any observed changes can be correlated with a single learning experience, and also because the time-dependent consolidation process can be delineated (Gibbs and Ng, 1977). Amnesia can be experimentally induced by giving trained chicks a brief transcranial electroshock, at an appropriate time after training so that any changes observed after training can be specifically correlated with memory storage processes and not to some other concomitant of the training procedure such as stress, arousal, or the perception of the taste of methylanthranilate. By this method, Rose and colleagues have found that the increased incorporation of ^3H -fucose (Rose and Harding, 1984) and the enhanced bursting activity (Mason and Rose, 1988) are specifically related to the learning of the task. These biochemical and electrophysiological experiments indicate changes at the pre- and post-synaptic level following the learning experience. In the electron microscopic investigation discussed above, Stewart et al,

(1984) have already reported alterations in the presynaptic area, but no studies in the chick have been performed after passive avoidance learning to find any postsynaptic correlates of information storage, such as that in dendrites and dendritic spines. The experiments reported here were designed to investigate this possibility and to determine whether or not changes in the number and shape of dendritic spines occurred in a particular class of neurons in the chick IMHV, 25h after passive avoidance training. The specificity of any observed change was also to be investigated, by rendering chicks amnesic with a brief transcranial electroshock delivered 5 min after the training.

The importance of dendrites and dendritic spines in information processing and their involvement in memory storage mechanisms, therefore forms the basis of this thesis. As such, the thesis will be separated into a preliminary discussion of dendritic spines, with a later chapter on dendritic branching, and a final discussion of their relation to memory storage in the chick.

CHAPTER 2

DENDRITIC SPINES

The application of the neuronal impregnation methods of Golgi (using silver) and Ehrlich (using methylene blue) in the late nineteenth century, revealed the presence of numerous small appendages on the neuronal processes, termed dendritic spines, in a wide variety of taxa. That these dendritic spines were not simply artifacts of the staining procedures, but true structural entities of the neuron was confirmed by the electron microscopic studies of Gray (1959). Structurally, dendritic spines appear devoid of mitochondria, and in the cortex of the mammalian brain, at least, occasionally possess a spine apparatus. A spine cistern is also sometimes found in the spine head, which is thought to be the intracellular store of calcium, much like the endoplasmic reticulum (Fifkova et al, 1983).

Interest in the nature and function of these structures grew primarily because initial empirical research had reported pathological changes in spine density and shape (for review, see Scheibel and Scheibel, 1968). For example, Berkley (1897) observed that with acute alcoholic poisoning, spines on rabbit pyramidal cells showed a marked decline in numerical density. The remaining spines appeared large and did not exhibit the rounded heads typically seen on the dendrites of normal cells. In a study of human neuropathological processes, Purpura (1974) described abnormally long and thin cortical dendritic spines (with the conspicuous absence of short, thick spines with mushroom shaped heads) in infants with seizures and mental retardation, compared to normal children of similar ages. The degree of dendritic spine loss and abnormality appears to be related to the age and severity of developmental retardation, rather than with dendritic geometry (Purpura, 1974; Marin-Padilla, 1972, 1974). In human Patau syndrome (D, 13-15 trisomy) one

case (newborn) showed thinner dendrites than normal and fewer spines on apical dendrites of upper layer cortical pyramids. The spines were also longer than normal newborns, which in turn are longer than in the adult, suggesting that the embryonic stage involving elongation of spines has persisted. Catala et al (1988) in studies of human neuropathology, recently reported decreased numbers of dendritic spines on cortical pyramidal neurons in dementia patients (Alzheimers, Parkinsons with dementia, Creutzfeldt-Jacob, Pick's, and dementia paralytica, cf. Catala et al, 1988) who also have memory dysfunctions, in line with other earlier reports showing similar changes in human Alzheimers (Mehraein et al, 1975) and alcoholic patients (Ferrer et al, 1986). Also, recent evidence for the atrophy of medium spiny I striatal neurons in human patients with Parkinsons disease has been presented by McNeill et al (1988). Such studies may directly correlate with cortical atrophy involving cell loss (of e.g. dopaminergic neurons, Hornykiewicz, 1982; Jellinger, 1986) and an increased number of astrocytes (Turner, 1968). These cortical changes will obviously result in decreases in the numbers of axons and interneuronal connections, which can lead to a reduction in the numbers of spines in particular brain regions because axons have a profound effect on the development and the subsequent maintenance of dendritic spines, as discussed below.

Whether such alterations in spine number and structure are causally related to learning performance and memory formation processes, and hence to mental disorders involving deficiencies in these behaviours, has been the subject of many investigations during the last thirty years. The major questions revolve around whether the cellular morphology of neurons and of dendritic spines is determined genetically, and is therefore a purely maturational process, or whether it is an *epigenetic* phenomenon, involving both genetic and environmental factors.

Answers to such questions will provide us with a better understanding of the nature of dendritic spines, and their relation to not only the normal physiological functioning of neurons within a network, but also to their importance in special processes such as those involved in learning and memory formation.

2.1. GENETIC BASIS.

Intrinsic factors (genetic) are important in determining neuronal cytoarchitecture as revealed by studies using cultured cells from snail *Helisoma* and vertebrate hippocampal neurons (Mattson, 1988). For example, embryonic *Helisoma* B5 and B19 neurons maintain their distinct structural identities in cell culture, the B5 neurons extending more complex neuritic arbors than the B19 types. Similarly, embryonic rat hippocampal pyramidal cells which have undergone their last mitosis, have sister neurons that are closely related morphologically: the axonal and dendritic arbors of one sister in a pair tend to be mirror images of those in the other sister.

In vivo studies of the genetic basis of spine and neuron growth have been based primarily on the cerebellum which has a highly ordered organisation, the interneuronal connections of which are well characterised (FIG. 1) (Llinas, 1975; Palay and Chan-Palay, 1974). The existence of mutant strains of mice in which there are gross morphological effects on the cerebellar organisation therefore allows good characterisation of the genetic and environmental factors regulating neuronal growth (Herndon, 1968; Herndon et al, 1971; Hirano et al, 1972; Llinas et al, 1973; Sotelo, 1975; Berry et al, 1980). The cerebella of mutant 'weaver' mice have no granule cells, and therefore no parallel fibres which are their axons, as part of a genetic defect (Hirano and Dembitzer, 1973; Rakic and Sidman, 1973; Sotelo, 1973). The parallel fibres in normal cerebella make synapses only on the dendritic

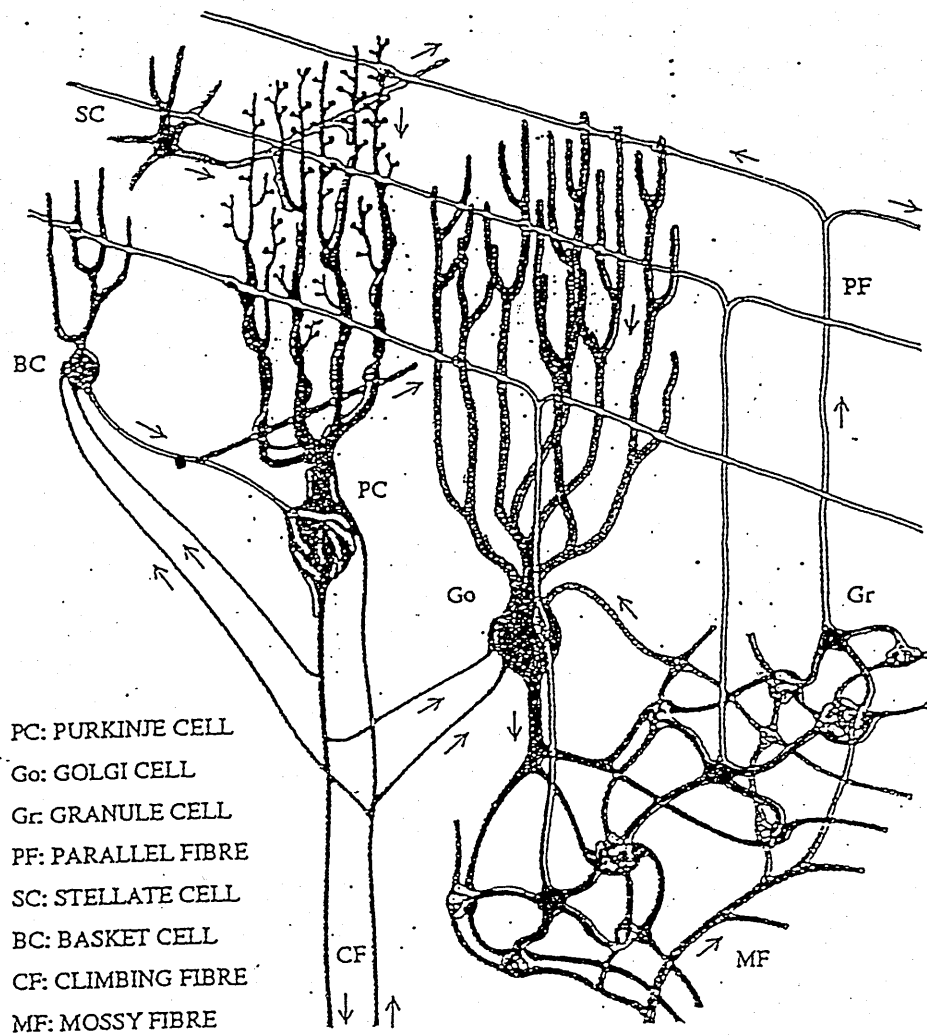


FIG 1. Connections between cells in the cerebellum. The connections of interest to the discussion in the text are those with the Purkinje cells, more specifically by the granule cell parallel fibre axons on Purkinje cell dendritic spines and the climbing fibre axons with the dendrite shafts and sessile spines of the primary dendrites (emanating from the cell body) of the Purkinje cells. In mutant weaver mice, the granule cells do not migrate to their final position, so that none, or very few, of the parallel fibres are present. Surprisingly, the tertiary dendritic spines of Purkinje cells still form but do have any synaptic contacts (From Llinas, 1975).

spines of Purkinje cell tertiary dendrites (Llinas and Hillman, 1969). However, weaver mice cerebellar Purkinje cells still have numerous dendritic spines in spite of the absence of any synaptic connections with parallel fibres. Also, these unattached dendritic spines have morphologically fully developed postsynaptic elements, although the postsynaptic thickening occupied a larger proportion of the spine surface than usual (Hirano and Dembitzer, 1973, 1974, 1975; Rakic and Sidman, 1973; Sotelo, 1973, 1975; Privat, 1975; Hanna et al, 1975). (This further substantiates the evidence discussed below for the existence of 'isolated postsynaptic densities').

Larramendi (1969) describes the involvement of dendritic spines in the *functional growth* of the Purkinje neurons. That is, during the development of the cerebellum, Purkinje cells form main dendrites which remain essentially stable, while small terminal, branchlets stretch out into the tissue to make particular parallel fibre connections. These branchlets, studded with spines, initially have no synaptic contacts in the upper molecular layer of 14 day old mice. However, when they are contacted by a specific parallel fibre axon, asymmetric type 1 synapses are formed with them, but only on spines. Larramendi (1969) further suggests that these dendrites grow spine-like processes, which, if they do not form synaptic contacts, develop into stems of new branchlets. Upon synapse formation, the spine-like extension stops growing and both the presynaptic bouton and the spine head enlarge. The synaptic contact length at this stage is rather long and shortens with maturity (a phenomenon which Larramendi refers to as "synaptic adhesion waning"). If this is the case, and the spine-like extension stops growing once a synaptic contact is formed, then one would expect a larger number of dendritic spines on Purkinje cells if the parallel fibres are absent. Chen and Hillman (1982)

show that this is indeed the case in the cerebellum of rats that have lost granule cells and parallel fibres after deafferentation.

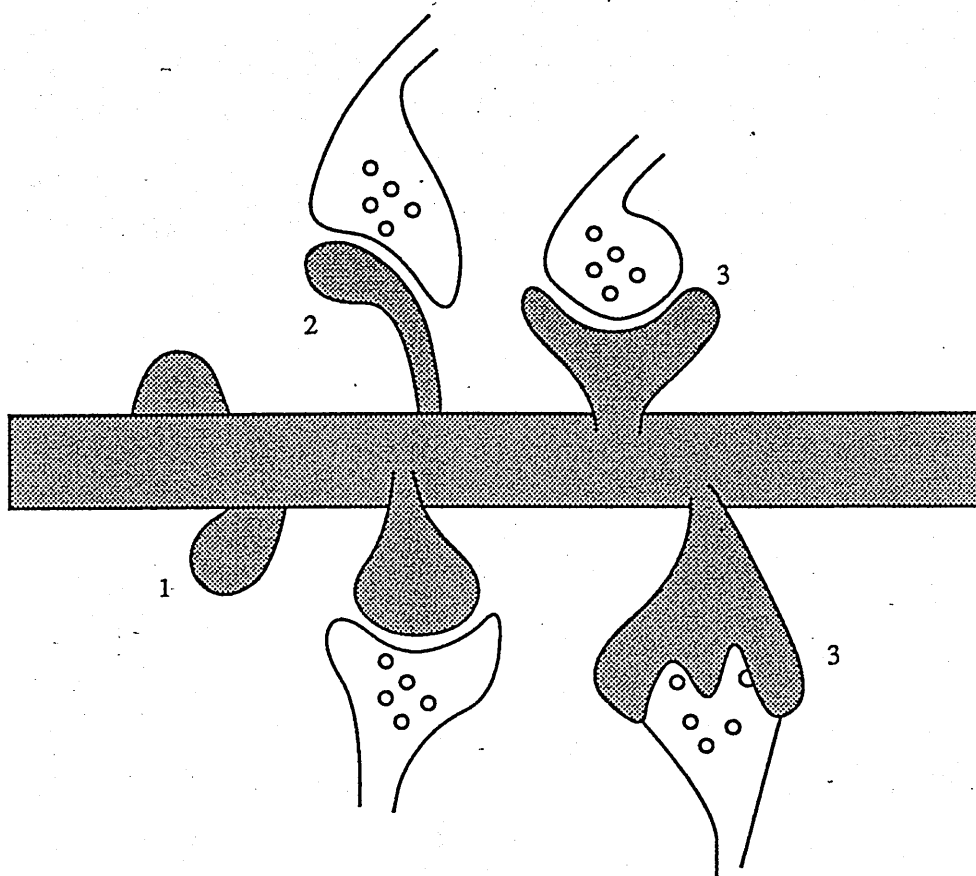
The Purkinje cells of the weaver cerebellum are often improperly orientated and elaborate only 12% of the normal number of dendritic segments (Bradley and Berry, 1978a), indicating an arrested development of the cells. Berry et al (1978) have identified the time of arrest at about the 7th day post natal, when parallel fibres normally begin to be laid down. Because in the weaver very few parallel fibres are present, it can be concluded that direct synaptic contact from day 7 with a critical number of parallel fibres is necessary for the further growth of the dendritic trees and spines. (The growth of dendritic trees and the involvement of afferents in this process is further discussed in chapter 5).

These studies show that in the cerebellum, the continued presence of presynaptic elements (in this case the parallel fibres) are unnecessary for maintenance of the dendritic spines, although they may be required for the induction of a *secondary* phase of growth of spines (Berry et al, 1980). Before this phase of induction, normal growth occurs in the weaver mouse cerebellum up to postnatal day 7. This growth may be either autonomous (Calvet et al, 1976; Gona, 1975), or directly induced by extracellular agents such as hormones, monoaminergic fibre activity (Berry et al, 1980; Rakic, 1974) or climbing fibre activity (Hamori, 1973). Hamori (1973) proposed an elegant hypothesis of an indirect, or heterotopic induction of dendritic spines by the afferent activity of climbing fibres synapsing on the Purkinje cell dendrite shafts. This hypothesis, however has been disproved by the observations of numerous dendritic spines on cultured Purkinje neurons which have no climbing fibre input (Calvet et al, 1974; Privat and Drian, 1974; Sotelo, 1975; Seil, 1979) or in vivo, where the climbing fibres have been destroyed soon

after birth (Sotelo, 1978; Bradley and Berry, 1976b). Indeed, the absence of climbing fibres seems to induce spine formation (Chen and Hillman, 1982; Sotelo, 1978; Bradley and Berry, 1976b), perhaps because in the normal cerebellum, climbing fibre contacts with the Purkinje cells causes spines on the cell soma and the proximal parts of the dendritic trees to regress (Bradley and Berry, 1976). If no climbing fibres are present then the spines will not regress and indeed continue to grow, hence giving the appearance of an induction of dendritic spines in the absence of climbing fibres. Thus, the development of Purkinje cell spines, and perhaps of spines in other brain regions, is an *epigenetic* property of the cell itself.

2.2. EXTRACEREBELLAR DEVELOPMENTAL CHANGES

Observations of dendritic spines on pyramidal and stellate cells of the adult striate cortex of many species have revealed the presence of type 1 synapses (asymmetric, rounded vesicles; excitatory actions) almost exclusively on the spine heads, with type 2 contacts (symmetric, pleomorphic vesicles; inhibitory actions) on the spine stem, dendrite shaft and cell bodies (Gray, 1959; LeVay, 1973; Colonnier, 1968; Mates and Lund, 1983). On the immature spiny neurons of the young animal, however, both type 1 and type 2 contacts are found on the dendrite shafts, suggesting that a redistribution of type 1 contacts occurs during maturation (Mates and Lund, 1983). Miller and Peters (1981) and Juraska and Fifkova (1979a,b) have investigated the development of pyramidal neurons of the rat visual cortex. Symmetric contacts are present on the dendrite shafts at postnatal (PN) days 1-3, but at days 9/10, synapses with both symmetric and asymmetric contacts are evident. Stubby spines with asymmetric contacts appear by day 6, and more complex spines with U shaped heads and thin stems (FIG. 2) become more frequent by day 15. Both studies report an increase in spine density from postnatal



1, STUBBY: $\bar{L} = 1 \mu\text{m}$
Range = $0.5 - 1 \mu\text{m}$

2, THIN: $\bar{L} = 1.7 \mu\text{m}$
Range = $0.5 - 4 \mu\text{m}$
Stem $\bar{L} = 1.1 \mu\text{m}$
Head = $0.6 \mu\text{m}$

3, COMPLEX OR
CUP SHAPED: $\bar{L} = 1.5 \mu\text{m}$
Range = $0.5 - 2.5 \mu\text{m}$
Stem = $0.8 \mu\text{m}$
Head = $1.4 \times 0.6 \mu\text{m}$

FIG. 2. Some shapes and lengths of dendritic spines. \bar{L} = average length of spine or stem. (From Peters and Kaiserman - Abramof, 1970).

day 7. In adult rats, stubby spines on layer III pyramidal neurons comprise only 19% of the total spine population and thin and mushroom-shaped spines (with asymmetric contacts) comprise 72% and 9%, respectively. A very similar developmental pattern has been reported by Cotman, et al (1973) for the dentate gyrus of the rat, a structure that essentially develops postnatally with a 100-fold increase in synapse density (number of synapses per unit area) from day 4 to adult. Also there is about a 50:50 ratio of symmetric to asymmetric synapses at days 4-11, when only stubby spines are predominantly found. This situation dramatically changes by day 25 and in the adult animal, when more complex spines of the U and W variety appear (see FIG. 2), almost all of which have asymmetric contacts on the spine heads. Mates and Lund (1983), studied the neuronal development of the macaque monkey striate visual cortex, and also reported similar maturational effects, although over a longer time course, on both pyramidal and spiny stellate neurons. In addition, Boothe et al (1979) have found a large increase in spine density on pyramidal and stellate cells of the monkey visual cortex from birth to week 8 and a subsequent decline to adulthood.

The development of asymmetric synaptic contacts essentially follows the appearance and growth of spines in all of the animals studied: monkey (Mates and Lund, 1983; Lund, boothe, Lund, 1977; Boothe et al, 1979; LeVay, 1973); cat and kitten (LeVay, 1973; Cragg, 1975a,b; Winfield, 1981) and rat and mouse (Juraska and Fifkova, 1979a,b; Ruiz-Marcos and Valverde, 1969; Feldman and Dowd, 1975; Valverde, 1971). The results of these studies show that the number of type 1 contacts, with corresponding dendritic spine densities, increase to a peak early in postnatal life (around eight weeks in monkey, six to eight weeks in cat, and 10-19 days in rat and mouse visual cortices) and a subsequent age-related decline. The

numbers of symmetric (type 2) contacts in mammals, however, tends to steadily increase in number to a plateau in adulthood (e.g. Winfield, 1981) and are always substantially lower in number in comparison to the type 2 contacts.

A similar developmental change in the density of spines and synapses has recently been found in the domestic chick (Hunter and Stewart, in preparation). The total synaptic density was found to have increased from embryonic day 16 (E16) to a peak in the left hemisphere at posthatch day 9 (P9), and then a reduction to day P22. The percentage of asymmetric synapses steadily increased during this period, such that there were 10% more asymmetric synapses at day P22 than E16, and about 8% less symmetric synapses, in both hemispheres. The percentage of spine synapses rises sharply from E16 to P1, to reach a plateau in both hemispheres, although the left LPO has a greater percentage of these types of synapses. Again, the percentage of shaft synapses decreases sharply from E16 to P1 to reach a stable level, with a significantly *lower* number in the left than in the right LPO. As in the mammalian studies discussed above, the increase in spine synapses is mainly due to *asymmetric spine synapses* (type 1 contacts) whilst the number of symmetric spine synapses and both symmetric and asymmetric *shaft* synapses decline with age.

These investigations show that not only is there a period of intense synaptic and spine growth (associated with type 1 contacts), but that there is a decline immediately following this "sensitive period". The central nervous system during the sensitive period is highly plastic and it is a time when the interneuronal organisation of the brain is at its most intense. Learning of specific behaviour patterns are also correlated with this period of development, e.g. Rehn et al (1986) and Apfelbach and Weiler (1985) have reported an increase in the development of spines on granule cells and synapses in the ferret olfactory bulb during the sensitive

period of prey odour imprinting at days 30-60 postnatal, with a subsequent decline. Olfactory granule cells in the rat also show an increase in the spine density up to day 21, with a subsequent reduction. Similarly, there is an increase in overall synapse density and in spine-synapse density in the IMHV and hyperstriatum accessorium (HA) up to 4 days posthatch (Bradley, 1985) and in the LPO (Hunter and Stewart, in preparation) of chicks up to 9 days posthatch. This increase coincides with the sensitive period of imprinting in chicks. Also, 1-year old mynah birds have about 50% of the spine complement found in 10-day old birds on large multipolar neurons in the hyperstriatum ventrale, pars caudale (Rausch and Scheich, 1982). This is thought to be related to the mynah birds' maturing ability to imitate sounds during the first year of its life (Rausch and Scheich, 1982). Developmental differences in the shape of spines have also been reported (see below).

Environmental manipulation also has a greater effect on the morphology of visual cortex pyramidal cells only if they are introduced during the early postnatal period and less, or no effect when performed in adult animals (Barlow, 1975; von Noorden et al, 1970; von Noorden, 1973). For example, during the sensitive period of development in the cat (from eye-opening to three months of age), monocular deprivation results in a drastic contraction of the deprived eye's sphere of influence in the visual cortex together with a compensatory expansion of that of the normal eye (Hubel et al, 1977). Periods of deprivation of a week or less are effective in eliciting changes; and in reverse suturing experiments, alternating the deprivation of the two eyes, results in the domination of the cortex, first by one eye, and then by the other. However, such deprivation has little or no effect outside this sensitive period (Hubel and Wiesel, 1970; Hirsch and Spinelli, 1971).

Further, Hirsch and Spinelli (1971) have shown that the physiology of the neurons in the visual cortex can be altered specifically in accordance with the visual input: rearing kittens with goggles enables specific visual stimuli to be delivered to each eye independently. One eye was presented with a view of horizontal bars, the other saw vertical bars. Nine weeks later, the receptive fields of neurons responding to the horizontally stimulated eye were found to be horizontal in their orientation; similarly the vertically stimulated eye produced vertical receptive fields. This apparent plasticity of the visual cortex argues against a strict genetic determination of the interneuronal connections within the region.

Morphological changes resulting from sensory deprivation, have also been found to be age-dependent. 30 days of monocular deprivation from the time of eye-opening significantly reduces the spine densities of pyramidal neurons in the visual cortex of rats. Little effect was found if older rats were deprived for the same length of time (Rothblat and Schwartz, 1979). Similarly Fiala et al (1978) found that environmental complexity only affected the branching patterns of hippocampal granule cells in young rats but not in adults. However, this is not to say that the morphology of neurons does not change in adult animals: the adult mammals are generally more resistant to the effects of sensory deprivation. Indeed, training young adult rats in a changing series of daily maze problems increases the upper apical dendritic fields of pyramidal neurons in the occipital cortex, compared to littermate controls (Greenough et al, 1979). More evidence for changes in the morphology of neurons in adults is presented in chapter 5.

The sensitive period in the chick is well known as being a time when the newly hatched chicks imprint on an object which has the "characteristics of a mother figure" (Immelmann et al, 1979; Hess, 1973; Scott, 1962). This period is important

with regard to the passive avoidance training paradigm used here as a model of learning, because the extreme plasticity of the brain at this stage would produce larger observable changes as a result of learning than would otherwise be visible. Therefore, this model will provide a better indication of the plasticity of the nervous system in response to learning and memory demands.

Thus, the early period of development is characterised by much interneuronal organisation, during which synapses and spines are mass-produced, and after which there is a decline. This decline could be due to many factors, including a loss of some of the contacts. Changeaux and Danchin (1977) have suggested that the decline in spine and synapse number soon after the termination of this sensitive period, occurs as a result of a process they have called "selective stabilisation". That is, some of the synaptic contacts will be stabilised and the "unused" contacts, retracted. It is unclear whether the mechanism of loss is active elimination of certain afferents or their passive withdrawal due to the absence of sufficient neural activity and/or (a possibly activity dependent) trophic factor. Some recent evidence for the influence of afferent fibres on the maintenance of dendritic spines has been provided by Nunzi et al (1987). They show that the normal age related dendritic spine loss in hippocampal CA1 pyramidal neurons can be prevented by the administration of phosphatidylserine, which can induce neurotransmitter synthesis and synaptic activity (Nunzi et al, 1987). Phosphatidylserine (PS) which is a component of brain cortical phospholipids, has been shown to stimulate brain catecholaminergic turnover (Toffano et al, 1978), and to increase acetylcholine output from the cerebral cortex of adult rats (Casamenti et al, 1979; Pedata et al, 1985). PS treatment has also been shown to enhance learning and memory functions in different animal models (Corwin et al, 1985; Drago et al, 1981; Zanotti

et al, 1986). These results suggest that synaptic activity may be a necessary factor in the stabilisation and maintenance of dendritic spines.

2.3. THE INFLUENCE OF AFFERENT FIBRES ON SPINE AND NEURONAL GROWTH.

From the above discussion, it is clear that neuronal organization is partially genetically controlled, and that the vertebrate nervous system is still in a sufficiently malleable state, especially during the early postnatal stages of life, that environmental influences can have a profound influence on its structure. The selective preservation, or stabilisation, of some of the synaptic contacts during early development has been suggested to be mediated by the level of activity of the afferent fibres (Changeaux and Danchin, 1977). There is indeed much evidence in the literature suggesting that afferent fibres are also involved in inducing the growth of dendrites and dendritic branches. Recently Black et al (1987) have reported a preliminary investigation showing that new patterns of neural activity, rather than mere cellular activity, are important for inducing changes in the morphology of neurons. They subjected groups of adult rats to one of four conditions: acrobatic conditioning (AC), where rats traversed a complicated elevated obstacle course that became progressively more difficult over the thirty day training period; voluntary exercise (VX), where rats had free access to a running wheel; forced exercise (FX), where the rats were subjected to a treadmill exercise routine; and an individual condition (IC) where the rats were kept in standard cages without the opportunity for additional exercise. The authors examined the paramedian lobule of the cerebellar cortex of the rats and found that the AC group, which learned more than the other groups, had a thicker molecular layer, a lower density of Purkinje cell somata with a correspondingly higher number of synapses per Purkinje neuron, in comparison to the other groups. Thus, the connectivity of the brain can be

influenced by specific patterns of neuronal activity and not simply by general cellular activity.

However, most of the studies in this area have been carried out in the visual cortex of various species of mammals, because of the ease with which the visual system can be manipulated and resulting changes in electrophysiological and morphological parameters, monitored. The degree of plasticity of the visual cortex is functionally very important for the adaptation of the organism to the environment: if the neuronal organisation of the brain was rigidly controlled by genetics, it would fail to respond to the unpredictability of the environment with which the organism has to interact and adapt.

Changes in the morphology of neurons and of dendritic spines in response to environmental manipulation and the direct involvement of activity in this process can only be inferred by correlational studies. This is because *in vivo* changes in cytoarchitecture are hard to monitor: light and electron microscopic investigations using fixed material and relying on statistical methods are perhaps the only means available for studies on this subject. Such correlational studies are abundant in the literature in the form of experiments dealing with visual deprivation and increased sensory stimulation.

There is considerable evidence from sensory deprivation, enrichment and deafferentation experiments, pointing to the importance of afferent fibres in the development of spines in various cerebral regions. For example, Shapiro and Vukovich (1970) show that 8 days of stressful stimulation of young rats from birth can increase spine density on cortical pyramidal neurons, and Globus et al (1973)

report increases in spine density of occipital cortex pyramidal cells after rearing rats in an enriched environment (social cages with toys changed daily) for 30 days. Also, continuous illumination from birth increases spine frequency beyond normal levels in both the lateral geniculate nucleus (LGN) (Parnavelas, Globus and Kaups, 1973) and the visual cortex (Parnavelas and Globus, 1976). In the LGN of the dog, where retinal efferents synapse on dendritic protrusions and spines of the relay cells, the dendritic spines fail to develop if the eyelids are sutured at birth (i.e. functional deprivation of the retinal ganglionic cell) even though the retinal afferents maintain synaptic contact with the parent dendrite (Szentagothai and Hamori, 1969).

In rodents, the spine frequency along apical dendrites of visual cortical pyramidal cells is reduced after postnatal dark rearing (Valverde, 1967, 1970, 1971) or monocular lid suture (Fifkova, 1968, 1970c; Rothblat and Schwartz, 1979). Valverde (1971) has shown that the rate of growth of normal pyramidal cell spine development in the visual cortex can be slowed when mice are reared in the dark from birth to 20 days. If the mice are then placed in normal lighting conditions for periods of up to 30 days, the spine density increases to control levels, suggesting that the development of the spine density on the apical dendrites of pyramidal cells depends on the arrival of normal visual inputs. The monocular deprivation studies, discussed above, suggest that these spine effects result from activity within the visual system rather than from hormonal or metabolic conditions, although the release of activity dependent trophic factors could also induce the same changes.

There is also a rapid increase in spine density in mice and rats (Valverde, 1971; Juraska and Fifkova, 1979; Globus and Scheibel, 1966; Cotman, Taylor and Lynch, 1973) at around the time of normal eye opening (day 15) and subsequently

during the sensitive period of development. This coincident growth of spines and eye-opening is also found in the guinea pig but with one difference: the guinea pig is essentially a precocial animal where the newborn can see, walk around and soon begins to eat solid food (Schuz, 1978). The mouse on the other hand only reaches this comparative stage of development at around the 13th day after birth.

Further, Bliss and colleagues (Zimmer, 1978), have shown that functional mossy fibres are present in the hippocampus of rats by day 5 postnatal; functional Schaffer collaterals by day 2 and functional perforant path fibres by day 7 postnatal (FIG. 3). Minkwitz (1976) reports that the spine densities of CA1 pyramidal neurons of the hippocampus increases 4-fold from days 5-10 after birth in rats, with an additional 2.5-fold growth from days 10-20. Thus it is possible that the growth phase of dendritic spines is somehow related to the perforant path activity or the mossy fibre activity from day 5 after birth. Deafferentation studies reveal that the presence of the afferent fibres is also necessary for the subsequent maintenance of the spines. Removal of commissural fibres (Stirling and Bliss, 1978) and perforant path axons by entorhinal ablation (Parnavelas et al, 1974), reduces the spine frequencies on dentate gyrus pyramidal neurons. An important finding is that although deafferentation of rodent brains can reduce spine frequencies, the effect can be temporary. Spine regrowth has been shown to occur in the hippocampus after deafferentation and subsequent re-innervation by invasion of axonal sprouts from other neighbouring regions (Parnavelas et al, 1974; Caceres and Steward, 1983). Interestingly, about 80-100 days after deafferentation in these experiments the spine density had essentially reverted to control levels. Indeed, following entorhinal ablation, fibres and synapses in the distal half of the dentate molecular layer degenerate and septal and commissural fibres sprout into the de-innervated

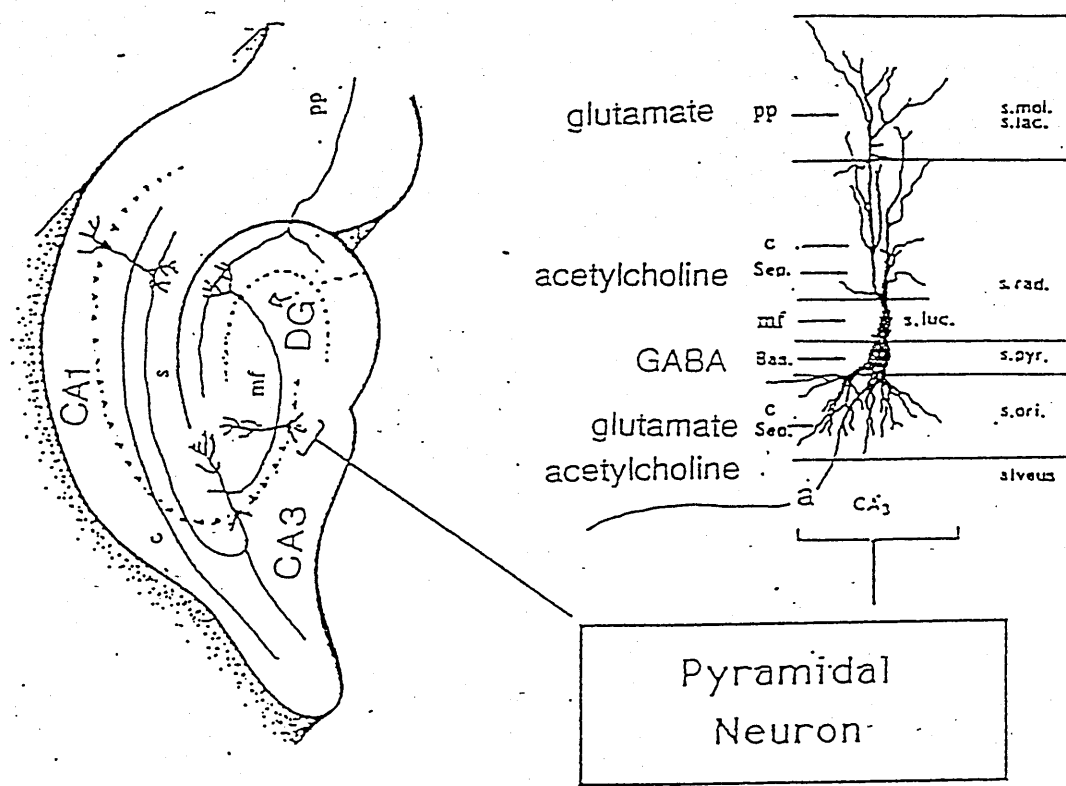


FIG. 3. Schematic diagram of the rat hippocampus and lamination of the neurotransmitter inputs to pyramidal neurons of field CA3. s, schaffer collaterals; mf, mossy fibres (glutamatergic); pp, perforant path (glutamatergic); c, commissural afferents; Sep., septal inputs (cholinergic); Bas., basket cell inputs (GABAergic).

area (Steward and Vinsant, 1983; Caceres and Steward, 1983). This suggests that spine regrowth and axonal sprouting may be causally related processes: the latter may somehow induce the formation of a new population of dendritic spines. This is similar to the induction of a secondary phase of growth of spines on Purkinje cells of the cerebellum which occurs upon innervation of the Purkinje cells by a critical number of parallel fibres. Thus, the altered activity in neuronal circuitry that occurs due to deprivation or changed activity around eye-opening clearly influences interneuronal organisation.

Some recent studies have indeed provided evidence suggesting that afferent activity can induce synaptic and spine growth and which can cause changes in the shape of the dendritic spines on the stimulated neurons, within a time period of about 15 min. For example, tetanic electrical stimulation of CA1 hippocampal neurons that can induce long term potentiation (LTP), causes an increase in the numbers of sessile (stubby) spine synapses within 15 min of the stimulation (Chang and Greenough, 1984). This increase can persist for up to 8h in the hippocampal slice without an overall change in synaptic density. Also Fífkova and van Harreveld (1977) have shown that such stimulation also enlarges the dendritic spines on pyramidal neurons of the dentate gyrus.

How can neural activity resulting from learning or patterned sensory stimulation cause changes in postsynaptic neuronal structures? The studies of LTP in hippocampal slices show that general hormonal or metabolic conditions may not be the only factors directly responsible for the observed morphological changes (Chang and Greenough, 1984). Mattson (1988) has suggested that it is possible for some trophic factors, including neurotransmitters released at the activated synaptic

site, to induce biochemical changes in the postsynaptic neuron which may eventually lead to morphological changes in the innervated neuron. In partial support for this, Hauser et al (1987) have shown that continuous blockade of opioid-opioid receptor interaction by opioid antagonists from birth to day 10 can increase the spine density of hippocampal pyramidal cells, granule cells in the dentate gyrus and cortical pyramidal cells. This manipulation also increases both brain and body size, and the number of brain cells of young rats. In addition, it accelerates the appearance of physical characteristics and spontaneous motor and sensorimotor behaviours (cf. Hauser et al, 1987). This study implicates the involvement of opioid receptors in the normal development of the brain. It is possible that the opioids have a normal role of inhibiting the release of trophic factors. Thus, the inhibition of the opioid receptors will result in the subsequent release of the trophic factors responsible for normal brain growth. This is only speculation and the exact mechanisms involved may well be more complex.

However, morphological changes may not occur every time neurotransmitters bind to the receptors: evidence in the literature suggests that both presynaptic activity and postsynaptic depolarization may be necessary for inducing changes in the morphology of the neurons (e.g. Hebb, 1949; Kelso et al, 1986; DiPrisco, 1984), or that the activity of a critical number of afferent fibres is necessary for changes of this nature to occur (discussed above). For example, LTP in the hippocampus can only be induced if the stimulus is of a sufficient intensity to coactivate a number of afferent fibres (Abraham and Goddard, 1984), and LTP producing stimulation causes an increase in the numbers of spine and shaft synapses (Chang and Greenough, 1984) and has the effect of enlarging the spines on the stimulated neurons (Fifkova and van Harreveld, 1977).

In terms of learning and memory processes where the association of several critical inputs to a given neuron will lead to the depolarisation of the cell, then corresponding morphological alterations may also result, such that the efficacy of those specific synapses will be increased (Hebb, 1949; DiPrisco, 1984). Thus, if the mechanisms by which afferent projections cause alterations in the morphology of target neurons is understood, then we would be in a better position to understand the processes involved in learning and memory. The hypothesis of the induction of a spine by a synaptic contact on the dendrite shaft, suggested by Chang and Greenough (1984) (and others), is therefore of importance in this context.

2.4. THE SPECIFIC INDUCTION OF SPINES.

Several investigators (Cotman et al, 1973; Miller and Peters, 1981; Mates and Lund, 1983 and Chang and Greenough, 1984) have proposed the idea that dendritic spines can be induced by the activity of a direct synaptic contact onto a dendrite shaft, such that the synaptic contact "pulls" the dendritic membrane out to form the spine (FIG. 4). This could explain why no spines have been reported to be synapse-free, and why about 80-90% of the synapses on mature neurons from the adult cerebral cortex, are found on dendritic spines (Peters and Feldman, 1976; Swindale, 1981; Gray, 1959; Colonnier, 1968). Electron microscopic investigations have shown that almost all of the spines have at least one asymmetric synapse, which is usually located on the spine head. The symmetric synapses are mainly found on the spine neck and dendrite shaft (discussed above). Miller and Peters (1981), Cotman et al (1973) and Mates and Lund (1983) suggest that spines may be induced by a symmetric synaptic contact on a dendrite shaft, which is later converted into an asymmetric contact on the spine head. In partial support of this hypothesis the investigators have quoted several reports which indicate that

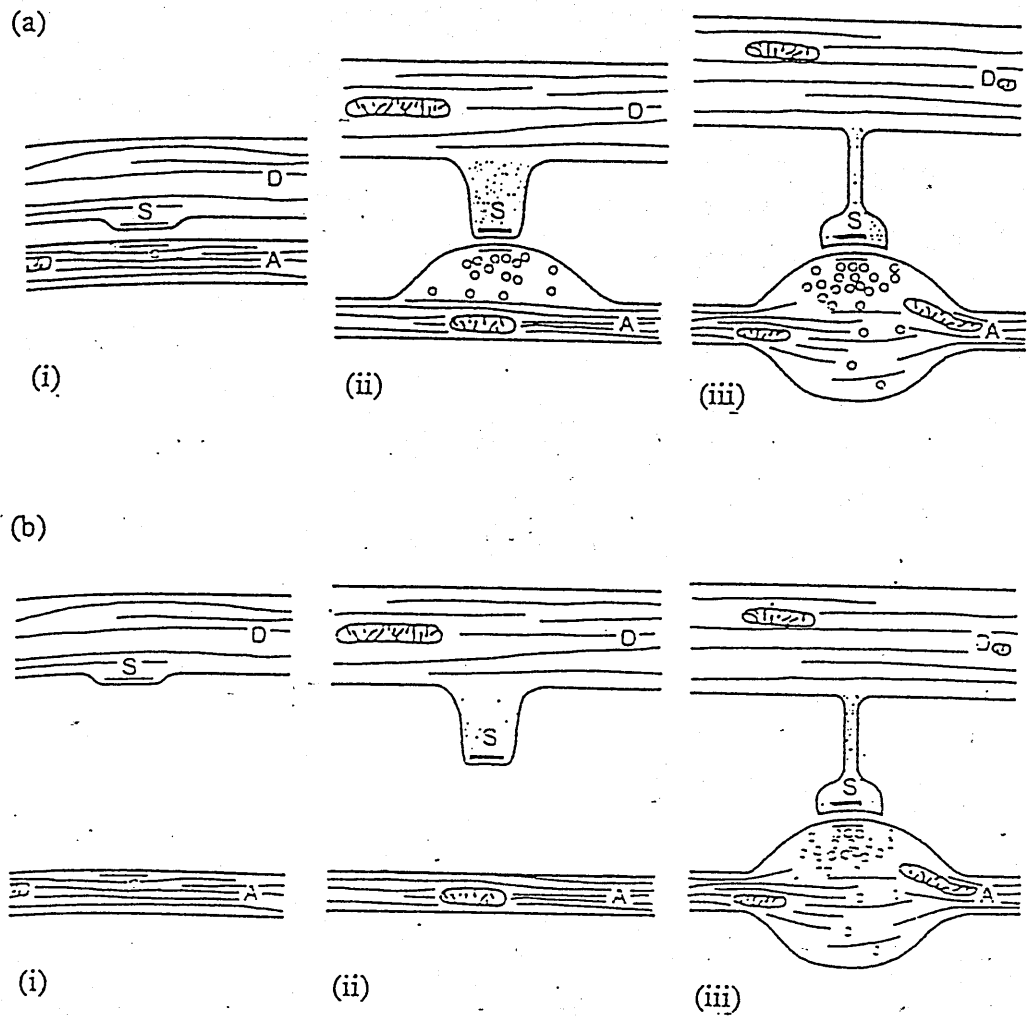


Fig. 4. Two modes of growth of dendritic spines shown as progressing with time [(i) (ii) and (iii)]: (a) the specific induction of a dendritic spine by a synaptic contact on the dendrite shaft. The presynaptic bouton swells simultaneously with the appearance of the spine-like swelling (Miller and Peters, 1981; Cotman et al, 1973; Mates and Lund, 1983; Lund et al, 1977). This process may be part of the mechanism of learning, as a means by which the efficacy of a synapse on the dendrite shaft can be altered (cf 'function of dendritic spines') (b) The growth of spines in the absence of any synaptic connection. This mode of growth occurs in cultured cells and in the cerebella of mutant mice (see text), and is indicative of either a genetic control of spine development, or an embryonic induction of spine growth. A = axon, D = dendrite, S = spine.

immature symmetric contacts can differentiate into type 1 synapses: Aghajanian and Bloom, 1967; Hinds and Hinds, 1976 and Rees et al, 1976. Hinds and Hinds (1976) further discuss the possibility that isolated postsynaptic densities (membrane attachments not having any apposing presynaptic dense projections or vesicles) are the direct precursors of symmetric or asymmetric synapses. They suggest that a thin postsynaptic density forms first, and then the presynaptic specializations form opposite it, giving a symmetric contact. Evidence for the existence of these isolated postsynaptic densities is numerous (see Hinds and Hinds, 1976 and Rees et al, 1976 for a further discussion and references therein; and the above discussion on Purkinje cell spines). In certain situations, however, asymmetrical synapses do not seem to pass through a symmetrical phase, as in early synaptogenesis of the cerebellar cortex of the rat, discussed above.

LeVay (1973) and Lund et al (1977) have extended this theory of spine induction one step further: that a reverse of the process can explain the age-related loss of dendritic spines and type 1 synapses on stellate neurons in the monkey visual cortex. That is, some of the dendritic spines may be retracted into the dendrite shaft, and the type 1 contacts upon them may possibly regress to the symmetric, type 2 form, which would then be located on the dendrite shaft. These authors use this argument to explain the case of *aspiny* neurons of the adult cat and monkey visual cortices. The neurons are heavily spined during the sensitive period of development in the young animal (see above), and display the characteristic synaptic distribution of type 1 synaptic contacts on spines (which appear 'headless'), and type 2 contacts on dendrite shafts. The adult varieties of these types of neurons are devoid of dendritic spines and have both type 1 and 2 contacts on the dendrite shafts (predominantly type 1) (LeVay, 1973, Lund et al, 1977). Moreover, dendritic

spines do not seem to persist in the absence of a type 1 contact: Lund and Lund (1970) examining type 1 contacts made by callosal fibres in area 18 of the adult rat conclude that dendritic spines of cortical neurons cannot survive deafferentation following degeneration of callosal fibres.

If learning and memory processes involve changes in the efficacy of certain critical synapses in an activated neural network, then one of the ways in which such a change may be brought about is if the synapses are located on a dendritic spine. By converting a shaft synapse into a spine synapse, the physiological properties of that synapse can be controlled to a large degree, because the strength of the signal transmitted across a spine can either be amplified or attenuated. This modulatory function of dendritic spines is discussed below at greater length (cf. "function of dendritic spines"). Thus, the direct induction of a spine by a synaptic contact on the dendrite shaft would be a very useful mechanism in the central nervous system.

2.5. SPINE SHAPE CHANGES.

Related to the previous discussion on the activity dependence of spine formation in certain neurons within various brain regions and animal species, is the evidence for changes in spine shape upon afferent innervation. For example, electrical stimulation of afferent pathways results in rapid spine swelling on neurons in dog and cat neocortices (Mikeladze, 1969). Similarly, a number of studies have found spine head enlargement (Desmond and Levy, 1983; Fifkova and Anderson, 1981; Fifkova and van Harreveld, 1977) and rounding (Chang and Greenough, 1984; Lee, Oliver, Schottler, and Lynch, 1981; Lee, Oliver and Lynch, 1980), after afferent projections to the hippocampus have been given brief trains of high frequency electrical stimulation. Such stimulation of certain hippocampal neurons is known to result in long-term potentiation (LTP) (Bliss and Lomo, 1973), although

the direct relation between changes in spine shape and LTP is still unclear.

Natural experiences also cause such alterations in spine morphology. Jewel fish with extensive social experience have long tectal interneuronal spines with markedly short stems, a condition that appears to result from an elongated swelling of spine heads (Coss and Globus, 1978). Narrow headed spines with longer stems have been observed in jewel fish reared under restricted environmental conditions (no social interaction and little environmental variation) (Coss, 1979; Coss and Globus, 1979), or prolonged juvenile crowding (Coss and Burgess, 1981; Berard, Burgess and Coss, 1981).

Changes in spine shape have also been found in a developmental context. In the granule cells of the ferret olfactory bulb, the mean spine stem length starts to decrease from day 60 postnatal, with a concomitant increase in spine head diameter (Rehn et al, 1986). This developmental change is thought to be related to the sensitive period of food odour imprinting in the ferret which occurs between days 30 to 60 postnatal. The number of spines and synapses also increases to a peak during this period and subsequently declines from day 60. Similarly, Rausch and Scheich (1982) noted a developmental decrease in spine density in mynah birds, with concomitant spine shape changes: the spines in 1-year old mynah birds had enlarged heads with thicker and shorter stems whilst those in 10-week old birds tended to have long, thin stems with smaller heads. The authors suggest that these changes reflect the mynah birds' maturing ability to imitate sounds.

More rapid alterations in the morphology of spines in the chick central nervous system have been reported, in the context of learning experiences. Bradley and

Horn (1979) reported that 21h old chicks exposed to intermittent periods of general illumination and a rotating flashing red light, the latter known to engender imprinting, had enlarged spine heads in the hyperstriatum accessorium, 24h later. This coincided in time with increased neural activity in the same brain region as measured by Brown and Horn (1979).

In honeybees, a week of extensive hive maintenance (from the time of emergence from pupation), results in spine head enlargement and stem shortening, which is further exaggerated among the longer spines after intense foraging activity for the following 2-4 weeks (Coss, Brandon and Globus, 1980). Similar changes have also been observed, although restricted to the longer spines, in one-week old honeybees after one-trial place learning which lasts only a few minutes (Brandon and Coss, 1982; Coss and Brandon, 1982). Burgess and Coss (1983) further reveal such spine shape alterations in jewel fish after 9min exposure to illumination, intermittent noise and an approaching net that engendered escape behaviour. These changes persist for at least 24h.

Clearly, alterations in spine density and shape can occur upon electrical and natural stimulation resulting from brief episodes of ecologically important behavioural activity and learning. Morphometric alterations can be induced within 5-9min which can persist for up to 24h, whereas an increase spine density has not been shown to occur in a similar time scale for the various behaviours mentioned, except with direct electrical stimulation (Chang and Greenough, 1984). The functional significance of changes in the number and shape of dendritic spines and their relation to learning and memory processes can now be addressed.

2.6. THE FUNCTION OF DENDRITIC SPINES.

The above discussion clearly suggests that dendritic spines are important neuronal structures and that they must subserve some significant functions. Computer simulation studies have provided data regarding the functional biophysical characteristics of spines and their importance in neuronal plasticity involved in learning and memory. Some possible functions of these distinct structures are considered below.

1) Dendritic spines can effectively modulate the amplitude of the excitatory postsynaptic potential (EPSP) of the synaptic input via the dimensions of its neck and head, and thereby have an important role in learning and memory mechanisms. Assuming passive propagation of the synaptic input on the spine head to the parent dendrite (cable theory), the current reaching the dendrite shaft at the base of the spine, will be attenuated: the thinner and longer the spine stem, the greater the reduction. This is because the shape of the spine is such that a narrow stem will have a larger cytoplasmic resistance than the spine head, thereby attenuating the signal reaching the dendrite shaft (Jack et al, 1975; Koch and Poggio, 1983; Wilson, 1984; Miller et al, 1985). If one assumes that the conduction of impulses through spines is not passive, but active, and involving the generation of action potentials, then theoretically dendritic spines could act as amplification devices (Jack et al, 1975; Miller et al, 1985; Perkel and Perkel, 1985). Perkel and Perkel (1985) show that incorporation of more active Na^+ or Ca^{2+} channels into the spine head membrane can amplify the synaptic signal, compared to the passive case, where the postsynaptic potential decreases almost linearly with increasing spine neck resistance. Miller et al (1985) have simulated the properties of spines with active membranes and conclude that the efficacy of a synaptic contact onto the head

of such a spine can be greatly enhanced compared to a synapse on the dendrite shaft or onto a passive spine. Placing an equivalent density of active channels on the dendrite shaft does not result in signal amplification; the electrical isolation provided by the spine neck is required (Perkel and Perkel, 1985). This would mean there is an optimal value for spine stem length and diameter (which determines the spine stem resistance), that can have the largest amplification effect. Rall and Segev (1987), Koch and Poggio (1983) and Miller et al (1985) have demonstrated this for various computed values of spine stem length and diameter and have found optimal ranges of spine stem resistance for which action potential generation is possible and for the maximal amplification of the signal conducted to the dendrite. Stretching the spine neck up to a specific size will have the effect of reducing the simulated somatic potential, whereas shortening the neck will increase the maximum depolarization amplitude reaching the soma. Thus, dendritic spines could theoretically function as synaptic EPSP amplifiers, if the membrane in the spine head was active, and if the values for the spine stem length and diameter were within the appropriate range.

2) Spines could theoretically increase the efficacy of distal synaptic inputs, by means of interactions between active, neighbouring dendritic spines (Shepherd et al, 1985; Rall and Segev, 1987). Shepherd et al (1985) have simulated spines on thin distal dendrites and show that an impulse in one spine head can trigger an impulse in a neighbouring spine head, again assuming active membrane properties. An important property they found for narrower spine stems or larger diameter dendrite branches (or both), was that several spines had to have received excitation in near synchrony, for the impulses to have generated sufficient current to trigger spines further along the dendrite. Also, faster impulse transmission to the cell body is possible in this manner, because the only sites assumed to have active, voltage-

dependent conductances were the spine heads, all other membrane was passive. Therefore, active propagation was discontinuous, resembling the saltatory conduction that takes place in myelinated nerve, where the conductance velocities are greater than for non-myelinated nerves. These are important observations regarding interpretations of the large spine density increases which have been observed following environmental manipulations of various sorts and learning (see above and chapter 6, the final discussion).

3) Inhibitory synapses were also found to be most effective at opposing the excitatory signals, when located on the dendrite shafts and spine stems (Koch and Poggio, 1983; Rall and Segev, 1987). Synaptic inhibition delivered to a particular dendrite branch was effective against all excitatory inputs along the same branch, and inhibition of the soma was effective against all excitatory inputs. Specific spatial and temporal control of the excitatory input is possible if inhibitory synapses are located on the spine stems or heads, thereby providing the maximum and the most effective information processing mechanism on the neuron (Koch and Poggio, 1983). Basic logic operations, including AND gates, OR gates and AND-NOT gates are therefore possible (Shepherd and Brayton, 1987). AND gates function to amplify the synaptic signals generated from two simultaneous excitatory inputs onto active spine heads at similar locations on the dendrite. This is also the case for the OR gates, where either one or the other of the inputs is active. But in this situation a larger initial excitation is needed to reach the threshold of depolarization in the single spine. AND-NOT gates provide specific control over excitatory inputs by the placement of particular inhibitory (hyperpolarizing) inputs onto either spine stems or dendrite shafts. Again, the excitatory inputs on the spine heads can be controlled by the constriction and expansion of the narrow spine stem. These operations are

especially effective when the inputs are located on distal dendrite shafts, because the interactions between active dendritic spines can theoretically enhance these inputs (Shepherd et al, 1985; Shepherd and Brayton, 1987; Rall and Segev, 1987).

4) Dendritic spines by their very structure, increase the surface area of the receptive field of neurons, thereby increasing the number of potential synaptic contact sites. This "connectionist" view of the function of dendritic spines suggests that spines grow out to meet axons that are perhaps distant from the cell body, which apparently happens in the Purkinje cells during development (discussed above). If the dendrite shaft was free of spines, then axons which are 3 or 4 μm from the dendrite shaft will have to re-route their course through the neuropile in order to make synaptic contacts with all of the relevant neurons (Swindale, 1981). The foregoing discussion on the epigenetic basis of the growth of dendritic spines supports this contention, in that new synaptic connections are rapidly forming soon after birth and this is also associated with an increase in the numbers of dendritic spines (see above).

In relation to memory storage mechanisms which may involve the formation of new synaptic contacts, one could postulate the growth of new spines that make connections with particular *en passant* axons. Also the activity of afferent fibres may induce the growth of spines throughout the neuron or specifically at a synaptic site.

2.7. CONCLUSIONS

Dendritic spines are an epigenetic phenomenon. Although some spines in early development are formed by genetic and maturational factors, hormones, neuronal activity and neurotransmitters have a profound influence on their growth and

subsequent maintenance. The intrinsic genetic development of spines probably initiates the first phase of spine growth, at which stage they probably do not have any synaptic contacts, and simply extend out into the neuropil to "meet" particular axons. This mechanism is more likely to occur in early development rather than in adulthood, because of the intense synaptic organisation that occurs soon after birth, especially during the sensitive period. The normal arrival of afferents, and their functional activity can induce the further growth of dendritic spines, the so-called secondary phase. This may require the presence of a critical number of afferents during early development to induce a general growth of spines throughout the neuron, thereby increasing the number of possible receptive sites on the neuron.

In adulthood and as a substrate for memory storage mechanisms, specific synaptic contacts on the dendrite shaft may directly induce the formation of a spine at that location as a means of increasing the efficacy of that synapse. Theoretically, spines can act as modulators of the synaptic information: the mere presense of a spine at the postsynaptic site has an effect on the physiological characteristics of a synapse on the spine head. Altering the dimensions of the spine stem and spine head can either attenuate or amplify the signal injected into the spine head, a property that has advantages and implications for the mechanisms involved in the storage of the learnt information.

From the arguments presented above, it is evident that dendritic spines can form within 15 min of direct electrical stimulation (Chang and Greenough, 1984) in *in vitro* slice preparations, and in response to various environmental manipulations *in vivo*, including learning. What is lacking in the literature, however, is the direct relation of dendritic spines to learning and memory processes, as opposed to the

general effects of arousal for example. Here it is proposed to ask:

(1) Do the memory formation processes for the passive avoidance training task involve changes in the number of spines? Are there also concomitant spine shape changes occurring as a result of passive avoidance training? Passive avoidance training is a one-trial learning task where long-term memory storage is believed to occur within 24h (Gibbs and Ng, 1977) and any changes in the number and shape of spines should be detected at this time point. The choice of a time point no longer than 24h, would be important for passive avoidance learning studies because confounding effects of imprinting may invalidate the results if the chicks are left for periods longer than 24h. Also, leaving the chicks for more than 24h in a controlled environment has practical difficulties. In addition, previous electron microscopic investigations were carried out at this time point (Stewart et al, 1984), so that any results from a light microscope study can be correlated with these.

(2) Can the dendritic branching patterns of identified neurons change as a result of the one-trial learning, and within 24h? Previous studies have only examined alterations in dendritic branching after several days to months of compound training (review: Greenough and Chang, 1985), e.g. maze-training for up to 30 days in rats (Greenough et al, 1979), and after operant conditioning for 7 days, also in rats (Mahajan and Desiraju, 1988) (see chapter 5). The dendritic branching studies and their relation to memory processes are discussed later.

(4) If any changes are found after training in any of these parameters, then we can also ask if they are specifically related to memory formation, rather than as a result of indirect influences from the training procedure itself, such as the effects of stress, arousal and of tasting the methylanthranilate *per se*. This can be

accomplished by using the electroshock procedure which renders trained chicks amnesic.

These hypotheses were tested by studying the changes in dendritic branching, spine density and spine shape on a class of large, multipolar projection neurons (abbreviated LMPNs, FIG. 5, 6, 9, 10, 11) in the IMHV, as opposed to small interneurons that had local axonal arborisations, within the vicinity of the dendritic field (local circuit neurons, FIG. 12) (Tombol et al, 1987). In a preliminary study, the latter neuron type did not show any qualitative change in spine density and was therefore not analysed here. Previous investigations of spine density changes under the light microscope also studied large, multipolar type neurons after auditory imprinting (Wallhauser and Scheich, 1987) for example, and in a developmental study of mynah birds which learn to imitate songs (Rausch and Scheich, 1982). The LMPNs in the IMHV regions of both hemispheres were studied here, because as mentioned earlier (and will be discussed again later), many of the biochemical, electrophysiological and morphological changes observed in this region, were lateralised.

FIG. 5. Photographs of two large multipolar neurons, each showing a long axon stretching out into the tissue. These correspond to type 2 projection neurons in the chick IMHV as defined by Tombol et al. (1987) and described here as large, multipolar, projection neurons (LMPNs). The scale bar represents 20 μ m in both pictures.

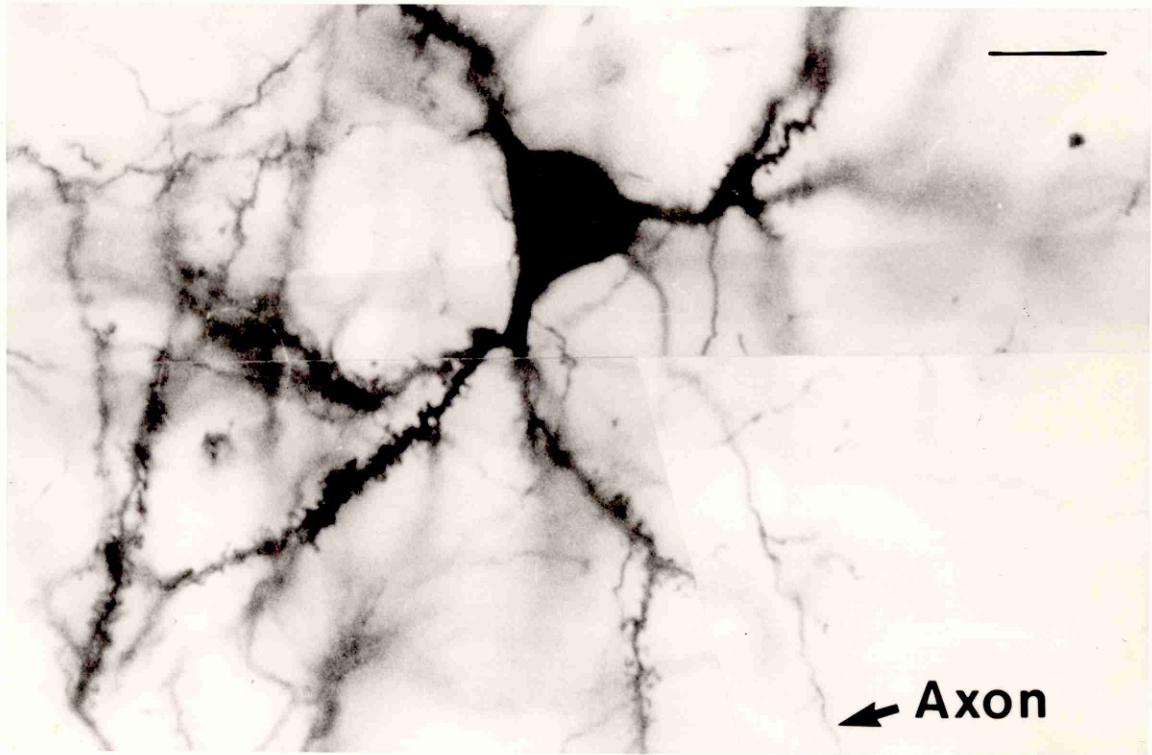
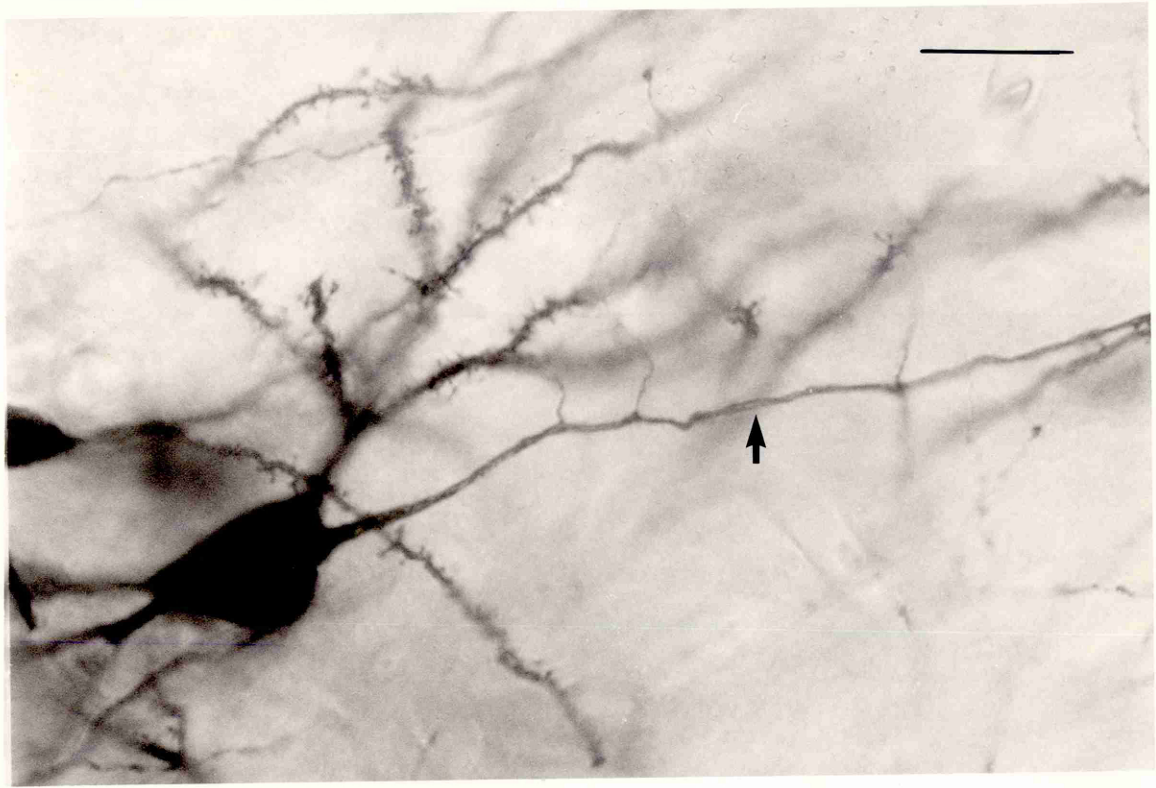


FIG. 5

CHAPTER 3

EXPERIMENT 1: A determination of changes in the number and structure of dendritic spines on large, multipolar projection neurons, 25h after passive avoidance training.

3.1 INTRODUCTION. The Golgi-technique was employed for the purposes of this experiment. The left and right IMHV regions from trained and control birds were dissected out from fixed brain tissue and Golgi-impregnated according to the rapid Golgi method. LMPNs were identified, and measurements were made under the light microscope. The training and experimental protocol are given below.

3.2 THE GOLGI-TECHNIQUE: PROBLEMS. The Golgi-technique itself has several problems, some of which have been outlined by Greenough (1984):

(a) *Golgi techniques fail to impregnate entire dendritic fields.* Some sections have dendritic processes with patchy staining. In some cases spines are visible in distal dendrites without, however, the dendrite being visibly stained. These sections were discarded from the spine analyses.

(b) *Dendritic fields may be partially obscured by other stained processes and are truncated by the section plane.* This is a major problem with regards to analysing all the dendritic processes of a given cell. Thus, only those dendrites clearly visible were analysed and those that were obviously truncated were not. Thicker sections (100-120 μm) in this respect, proved to be of value.

(c) *Golgi stains are capricious, staining a small fraction of neurons, and experience conditions or other biasing factors could affect which neurons are impregnated.* It is well known that not all of the cells are impregnated by the Golgi

method, but what is unknown is why this should be the case. It is difficult to decide whether the staining pattern results from the neuronal activity due to the experimental manipulations or whether it is a purely random process. For the purposes of these experiments I have assumed that the staining process is random, in line with the conclusions of Pasternak and Woolsey (1975). These problems notwithstanding, many studies using Golgi-staining for a variety of different purposes have produced fairly consistent staining patterns of nervous tissue in the vertebrate central nervous system. Such data have been successfully used in numerous quantitative and qualitative investigations of neuronal and dendritic morphology and has been chosen here as a suitable method.

3.3 ESTIMATION OF THE TRUE TOTAL NUMBER OF SPINES.

One of the problems of counting the number of spines in Golgi-stained dendrites has been enumeration of only those spines protruding from the shafts into the clear zones flanking the dendrite. Feldman and Peters (1979) have presented a technique for estimating the total spine numbers on Golgi-impregnated dendrites which is relatively accurate. The most accurate technique at present employs reconstruction from serial thin sections of dendrites oriented in a plane transverse to the dendritic axis. The limiting factors of this technique are the numbers and abbreviated lengths of dendrites that can be comfortably analysed. In addition, it is usually difficult with this method to specify unequivocally the identity of the dendritic segment examined.

A second technique which also has several drawbacks, involves de-impregnating the Golgi-impregnated dendrites using the procedure of Fairen et al. (1977) i.e. gold-toning (see "future directions"). This procedure allows assessment of large numbers and longer lengths of dendrites since it makes the dendrites relatively translucent, thus revealing the spines previously hidden by the opacity of the Golgi-

impregnated dendrites. The major drawbacks of this method are (1) the difficulty in obtaining a sufficient number of suitably de-impregnated examples, (2) some of the spines themselves may not retain enough dense precipitate to remain visible in the light microscope, and (3) deimpregnations frequently leave fine diameter shafts too dark for light microscopic assessment of total spine number (see FIG. 6).

Thus both techniques have limited usefulness as routine spine-counting methods. Although many other attempts have been made at circumventing the difficulties inherent in visible spine-counts, the most useful technique in estimating the total spine number on Golgi-impregnated dendrites seems to be that presented by Feldman & Peters (1979). Their method is now described and was used in this study.

Firstly, several assumptions are made which, in practice, may not be perfectly satisfied but are nevertheless reasonable and have to be made, since this is a geometrically-based technique. Amongst these assumptions are the following (Feldman and Peters, 1979):

(a) Dendritic shafts may be considered to be cylindrical in form and of constant diameter over the dendrite length studied. Obviously, neuronal dendrites are not perfectly geometrical, due to areas of flattening and kinking of the dendritic shaft. It is difficult to correct for this and it does not seem to affect the accuracy of the technique significantly.

(b) The surfaces of the cylinders representing the dendritic shaft and the domain into which the spines extend are concentric.

FIG. 6. Golgi-gold toned neurons. *Top*: A low magnification photograph of a gold toned LMPN (arrow), which has the axon still visible under the light microscope. *Bottom*: A Golgi-gold toned LMPN at higher magnification, still has spines which are just visible. Unfortunately, some of the dendrites are still too dark for light microscopic assessment of total spine number.

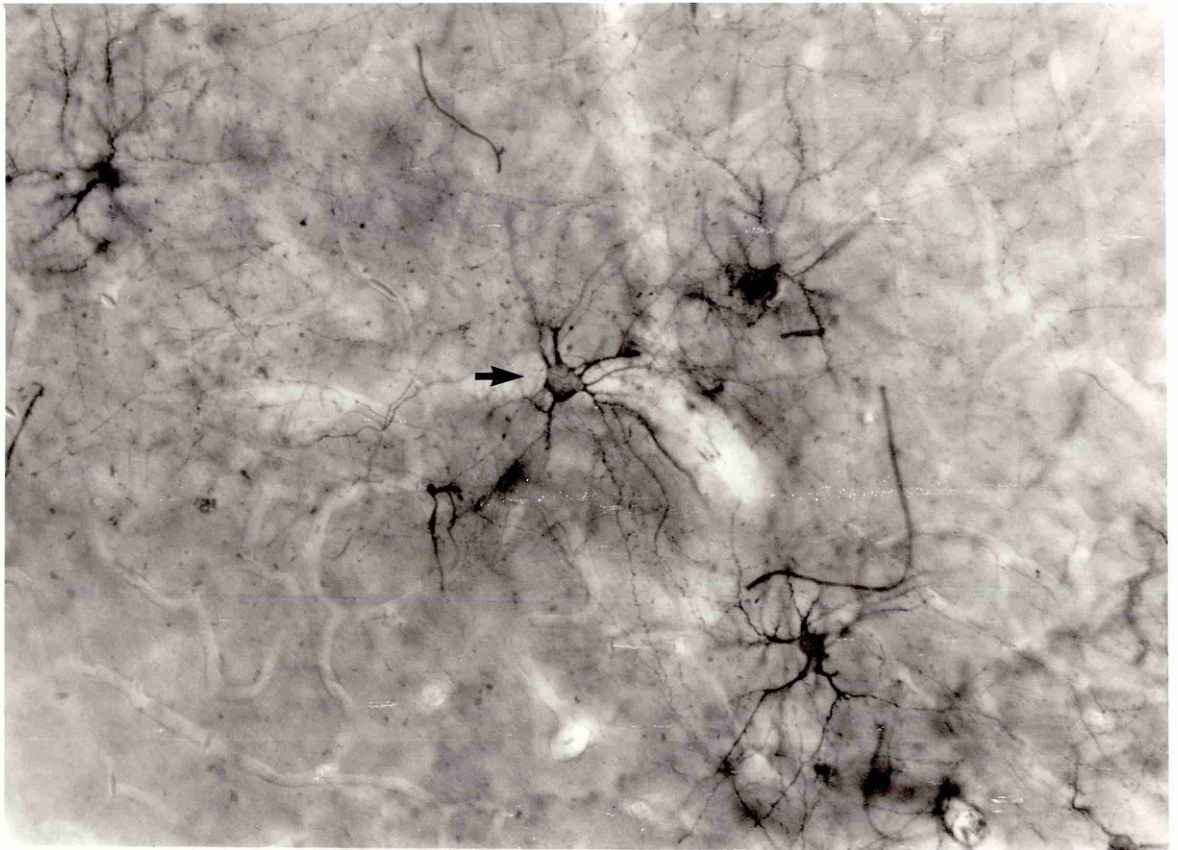


FIG. 6

(c) All spines whose tips project into the visible flanking zones by a distance equal to or greater than the average diameter of the spine heads will be included in the visible spine count. It is assumed that spines projecting into the flanking zones by less than this amount will not appear as unequivocal spines and will therefore not be counted.

(d) Dendritic spine origins are randomly distributed over the entire surface of the dendritic shaft segment examined.

This assumption, and the others, have been tested experimentally by Feldman & Peters (1979) by electronmicroscopically analysing serially sectioned dendrites of approximately 5.5-9.5 μm in length. They conclude that these assumptions are acceptable.

3.3.1 DERIVATION OF THE FORMULA

The two most significant variables which determine the relationship between the visible spine count and the true total number of spines are the dendrite shaft diameter and the spine length. The thicker the dendritic shaft, the larger the number of spines will be obscured by the opaque impregnated shaft, thus underrepresenting the total number of spines. Longer spines will more likely protrude more into the visible flanking zones and thus be included in the visible spine count.

The method is based on the ratio (FIG 7):

$$\frac{n}{N} = \frac{A_c \text{ (the total area of the left and right zones containing the visible spines)}}{A_s \text{ (the total area of the zone around the dendrite containing all the spines present)}}$$

FIG. 7. Schematic representation of a dendrite shaft of radius D_r , shown on the left in a three dimensional longitudinal view and on the right in cross-section. Spines protruding from the dendrite shaft are not shown, but concentric cylindrical regions one of wall thickness SD and one of wall thickness SL are presented. In spine counting of Golgi-impregnated dendrites viewed from the direction of the arrow, only those spines which extend into the shaded regions $Ac(1)$ and $Ac(2)$ will be visible. See text and appendix B for further details of the derivations of the formulae (Feldman and Peters, 1979).

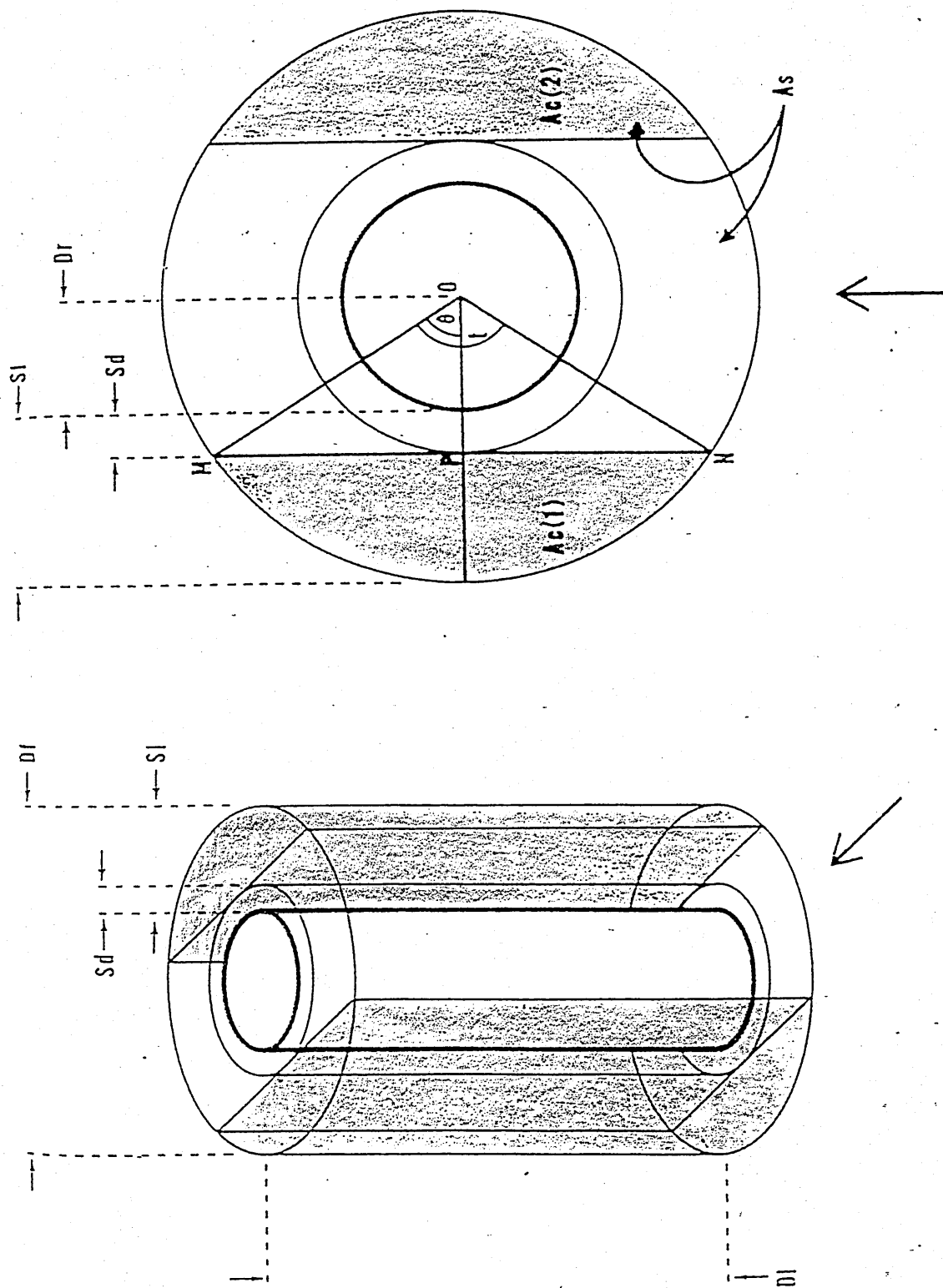


FIG. 7.

Where n = number of spines visible on the zones flanking the Golgi-impregnated dendrite, and N = the true total number of spines on the dendrite.

Actually, the ratio should be between volumes rather than areas, but since the volumes are calculated using the areas multiplied by the dendrite length (which is a constant factor), one can just as well use the areas rather than the volumes in estimating the total spine number. From the ratio the estimate of N can be calculated:

$$N = \frac{n A_s}{A_c}$$

This is assuming that the ratio between the numbers of visible (n) and total spine tips (N) will be the same as the ratio between the area of the zone containing the visible spines (A_c) and the area of the zone containing all the spines present (A_s) (FIG. 7). After making geometrical computations for the various areas the resulting final formula for N is thus, (see the appendix in Feldman & Peters (1979) and appendix B of this thesis for the details of the computations).

$$N = \frac{n\pi[(Dr+SL)^2 - (Dr+SHD)^2]}{2\left[\frac{\theta}{90}\pi(Dr+SL)^2\right] - 2(Dr+SL)(Dr+SHD)\sin\theta}$$

3.4 METHODS

The general training, perfusions, and measurements were similar for all of the studies using the Golgi staining technique, described in this thesis. This chapter, therefore, gives a detailed account of the basic Golgi technique used and will be referred to again in later chapters.

3.4.1 TRAINING. In each replication of the experiment eight, one day old chicks (Ross I chunky; incubated and hatched in communal brooders, at 38-40°C, on a 12h light/dark cycle) were placed in pairs in pens (20x25x20cm) with red overhead illumination (60W) at approximately 09:00 hours. The chicks were allowed to settle for one hour and were then pretrained twice (with a 10 min interval) on a small (2.5 mm diameter) dry white bead to encourage pecking. 10 min later, four out of the eight chicks (=2 pens) were presented for 10 sec with a shiny (4mm diameter) chrome bead coated with methylanthranilate. These chicks pecked once and immediately showed a disgust response (head shaking, wiping the beak on the floor of the pen etc.) and avoided the bead thereafter. The control chicks were offered a non-aversive, water coated bead which they freely pecked. The chicks were then tested ~30min later by presentation of a similar but dry bead. None of the M-chicks pecked at the bead, while the W-chicks pecked and continued pecking. Occasionally one or two chicks failed to respond to the training - controls may avoid the bead or the trained chicks may peck at it, but this was rare.

3.4.2 PERFUSION. The chicks were kept in their pens under continuous illumination for 24h and re-tested on a dry chrome bead, 10-15min prior to perfusion (all perfusions commenced 24h after training). Only those chicks showing the correct response - pecking if controls and not if trained - were used for further experimentation, and of these, 2M- and 2W-chicks were selected for Golgi

studies because only 4 chicks could be perfused on a single day (to keep the training to perfusion interval at 24-26h, because perfusing just one chick can take up to 30min). The chicks were given an overdose of sodium pentobarbitone (usually ~0.2ml) and perfused through the left ventricle of the heart with 10-15ml of 0.9% saline pre-fix followed by 100-125ml of an aldehyde fixative (1% paraformaldehyde, 1% gluteraldehyde, 0.003% CaCl_2 in 0.12M phosphate buffer, pH 7.3). After 20-25 min of perfusion the brain was removed and kept in the same fixative for 24-48h. Small blocks of the left and right IMHV regions were dissected out (FIG. 8) using a specially prepared brain mould (Rose and Csillag, 1985). In all a total of 9 M and 8 W chicks were used.

3.4.3 RAPID GOLGI PROCEDURE. The tissue blocks were incubated in 1% OsO_4 , dissolved in washing medium (8% glucose and 0.003% CaCl_2 in 0.12M phosphate buffer) for 5h, washed quickly and left in 3.5% $\text{K}_2\text{Cr}_2\text{O}_7$ for ~48h. The blocks were then briefly washed in 0.75% AgNO_3 and kept in fresh 0.75% AgNO_3 in the dark at room temperature for 12-18h. They were then embedded in 7% agar at ~37°C and cooled to 4°C before cutting into 90-120µm thick sections with a Sorval tissue chopper (this variation in section thickness was due to problems with the tissue chopper, but most of the sections were about 120µm thick). The sections were temporarily mounted onto slides with absolute glycerol and later embedded in DPX mountant (the edges of the coverslips were sealed with clear nail varnish in order to prevent the DPX from 'shrinking'). Measurement of the variation in section thickness was obtained and checked by selecting a range of sections, some of which were obviously very thick due to their opacity, and others which looked very thin. These were re-embedded in agar, and cut in cross-sections. The cross-sections of the tissue were then measured accurately using a calibrated ocular

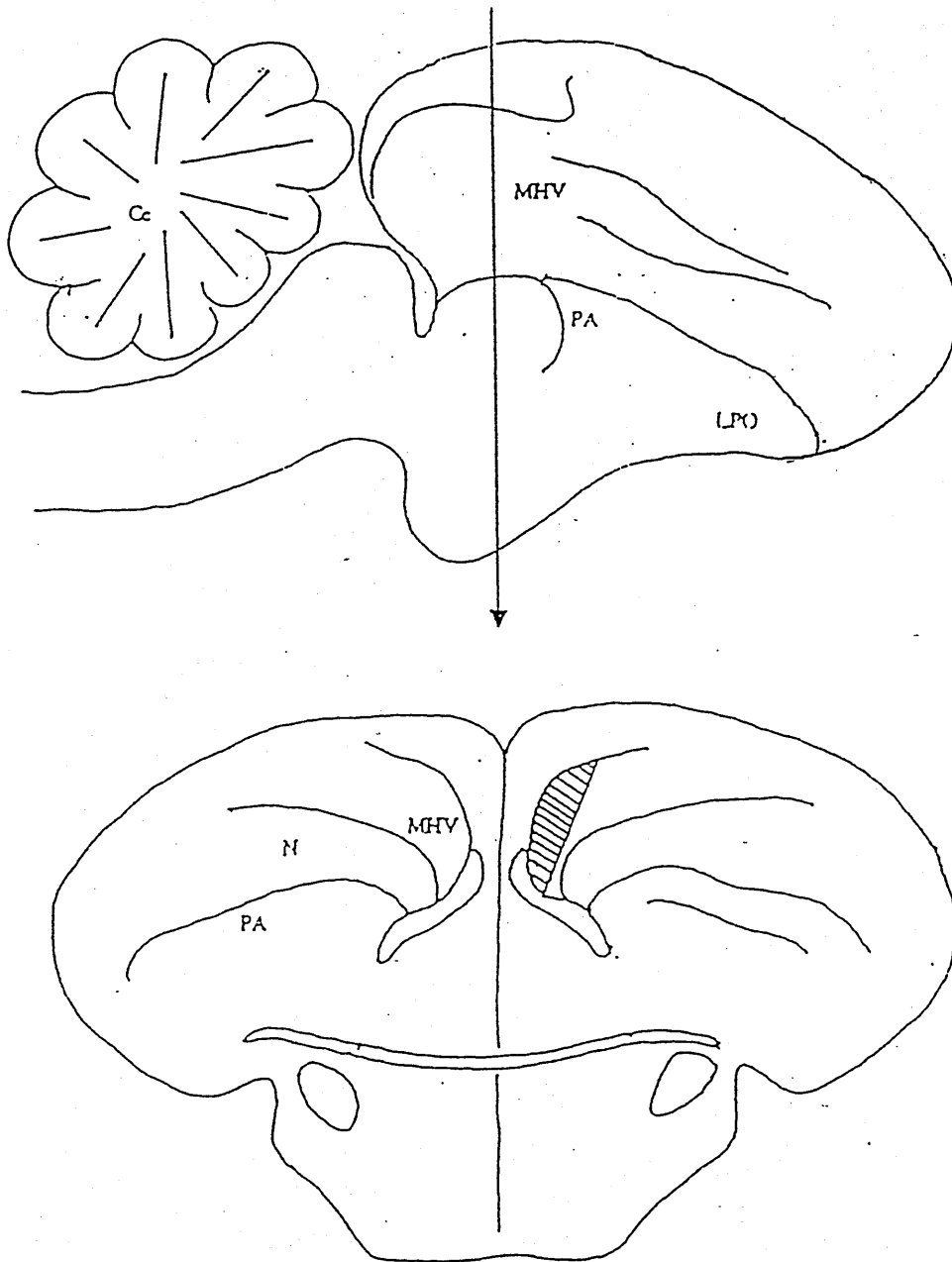


FIG. 8. (a) Schematic diagram of a longitudinal section through the 2-day old chick brain, and (b) the cross-section made using a brain mould to dissect out small blocks of the IMHV, shown here as the shaded area. IMHV, intermediate medial hyperstriatum ventrale; PA, paleostriatum augmentatum; N, neostriatum; LPO, lobus parolfactorius; Ce, cerebellum.

graticule on a Zeiss light microscope.

3.4.4 MEASUREMENTS. The slides were coded so that all measurements were carried out "blind". The region of the IMHV studied lay close to the ventricular surface within an area of $\sim 0.75\text{-}1.15\text{mm}^2$ (FIGS. 9, 10). The following criteria were used in the selection of cells for measurement; the cells were *large* (maximum diameter of the soma being between 17 and $25\mu\text{m}$), *multipolar* (with three or more primary dendrites emanating from the soma), and "*projection*" in nature, i.e. having long axons that project away from the immediate vicinity of the neuron) (FIGS. 5, 6, 9, 10, 11, 12). These cells correspond to type 2 projection neurons as classified by Tombol et al, 1987). Here they are referred to as large, multipolar, projection neurons (abbreviated as LMPN's).

Further, only those neurons which were well impregnated and for the most part, unobscured by other processes were measured.

The dendrites of identified cells were ordered centrifugally according to the method of Coleman and Riesen (1968) which orders branches away from the cell body towards the periphery (FIG. 13). This method of ordering was favoured over the 'centripetal' form, which orders the dendrites from the periphery inwards toward the cell body. Thus, by the centripetal method, the terminal branches in a tree will be designated as the first orders. Two first orders drain into a second order, and so on. One of the major problems with the centripetal method is that it can give erroneous labelling of the dendrites if the terminal branches are truncated by tissue sectioning. The ordering of the entire tree will change if the actual terminal branches are truncated. The centrifugal ordering system does not suffer from these effects,

FIG. 9. *Top*: Low power photograph (x78.75) of a typical Golgi-stained section, showing the ventricular edge (arrow). *Bottom*: Another Golgi stained section taken from a control chick, at higher magnification (x126), showing a LMPN in the centre. Selection of cell types was conducted at these magnifications. Higher magnifications were used for verification and spine and dendrite measurements.

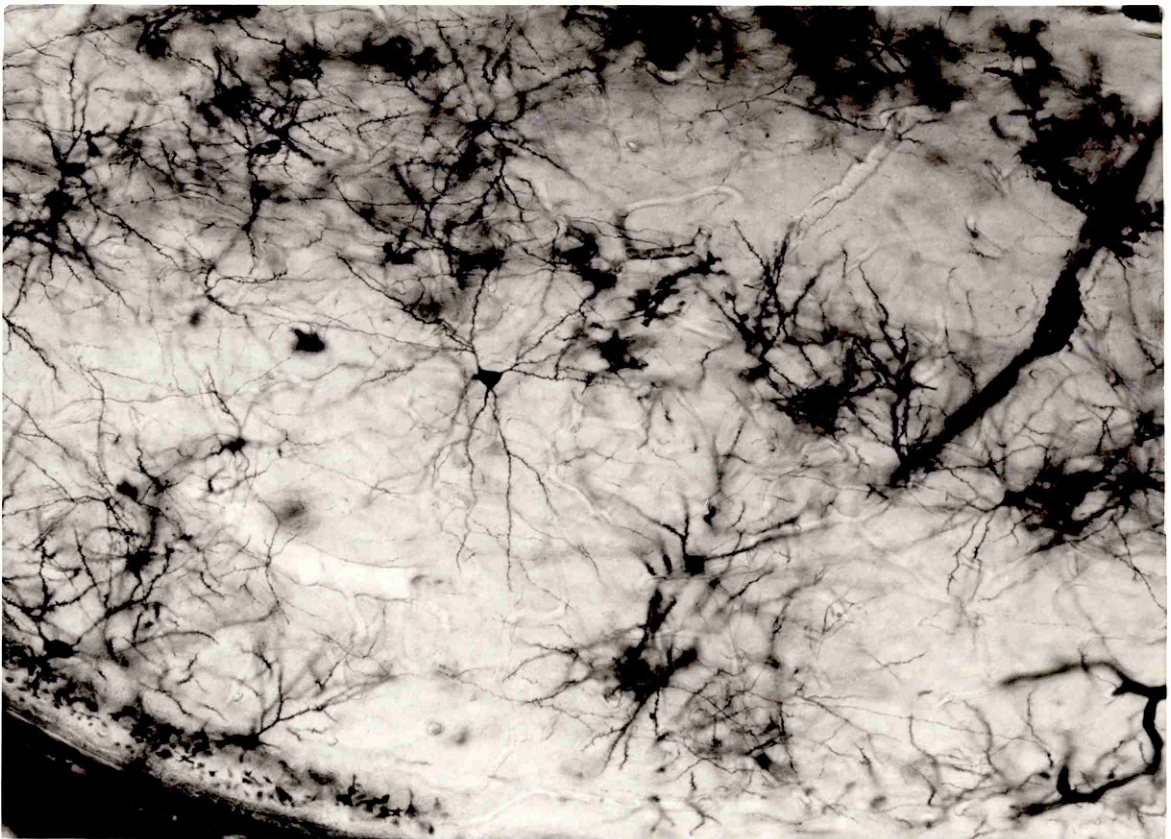
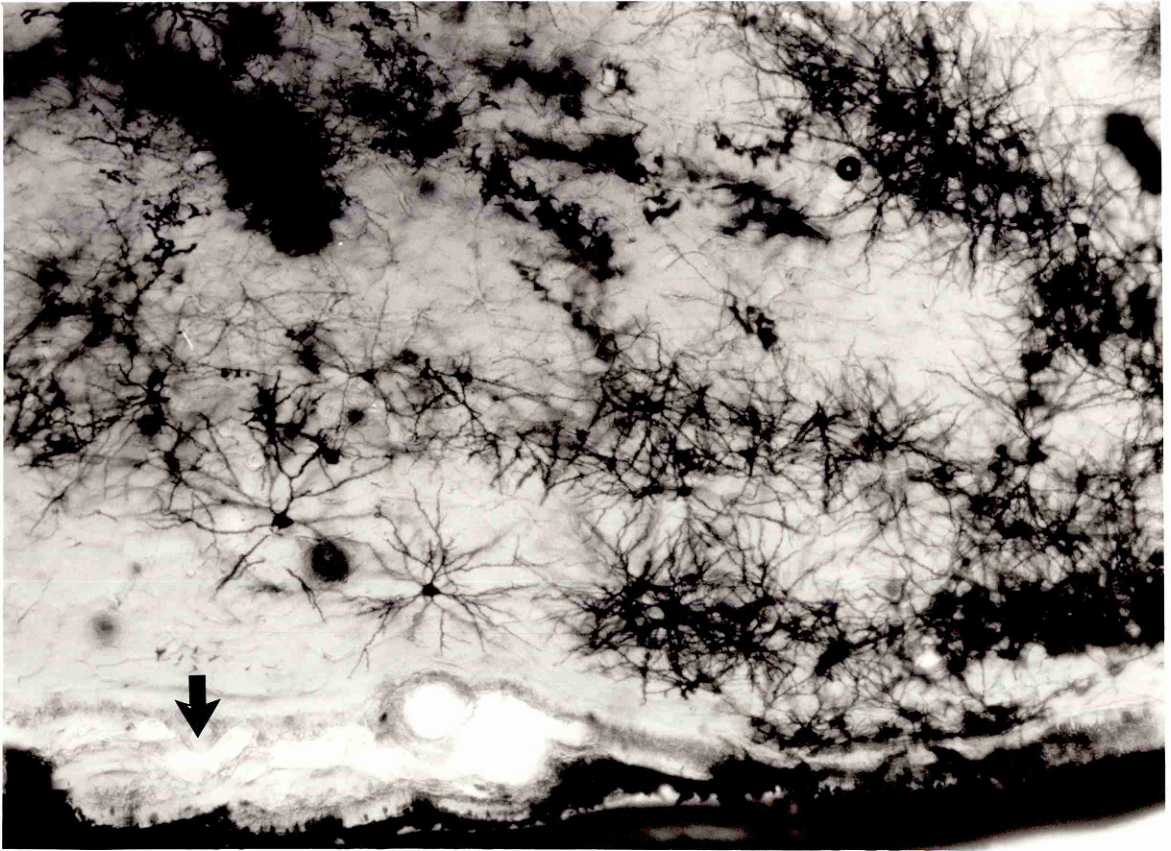


FIG.9

FIG. 10. *Top*: Golgi-stained tissue showing cells with broken dendrites (arrow). Although, the staining was very good in other respects, these sections were discarded from the analyses. *Bottom*: A small, aspiny neuron (asterix) and a LMPN which is out-of-focus, to make the axon visible (arrow).

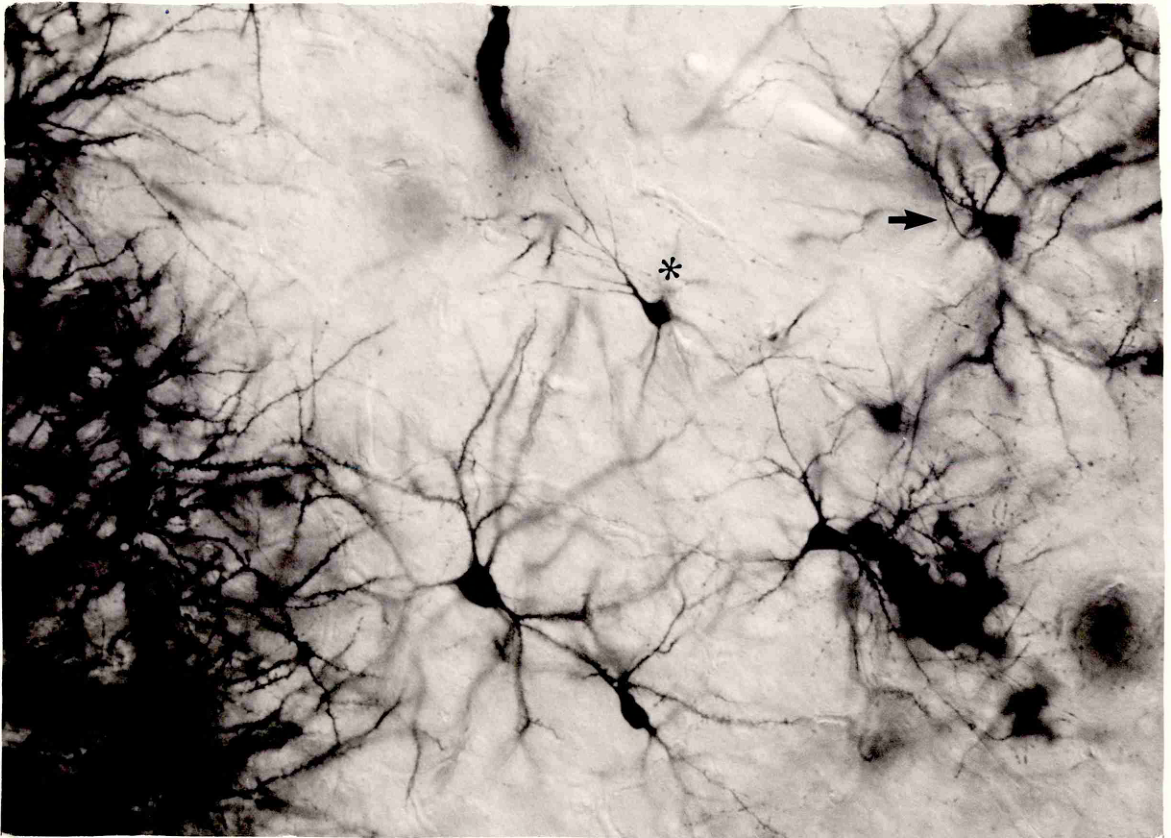
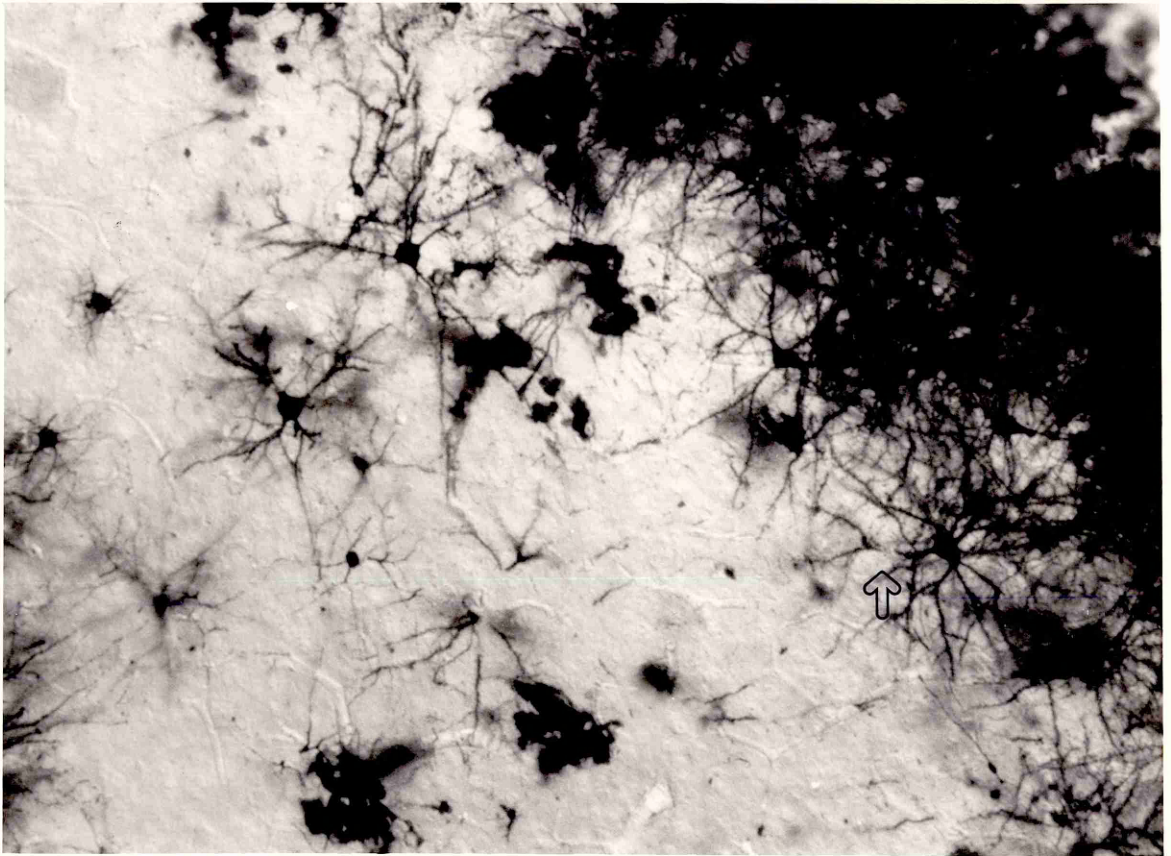


FIG. 10

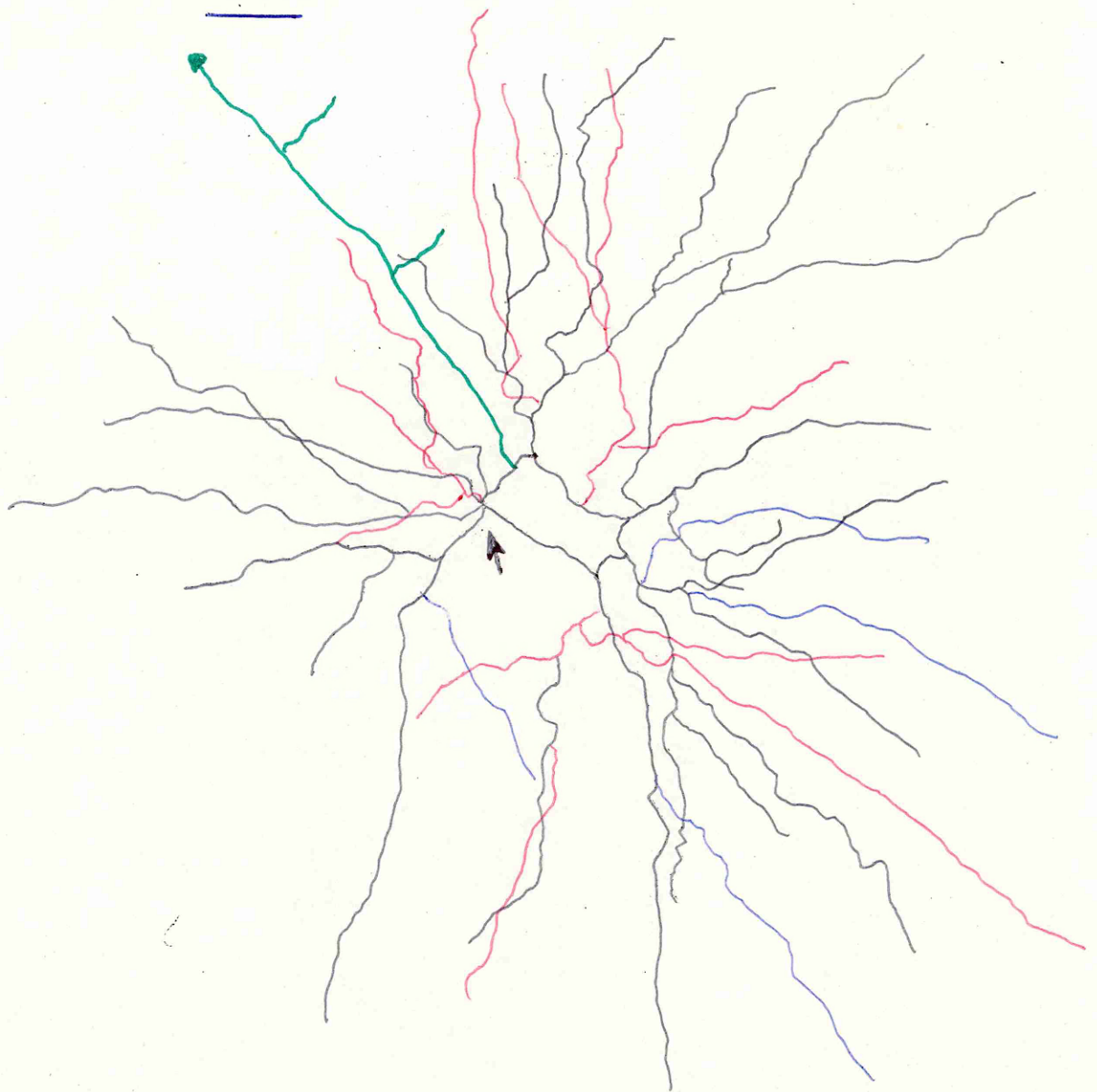


FIG. 11. Camera lucida drawing of the dendritic trees of a LMPN from the left IMHV of trained chicks. The dendrites are drawn with different colours to enable the dendritic trees to be differentiated. The arrow points to a quadchotomous vertex (see chapter 5, FIG. 23), and the axon is shown in green. Scale bar = 20 μ m.

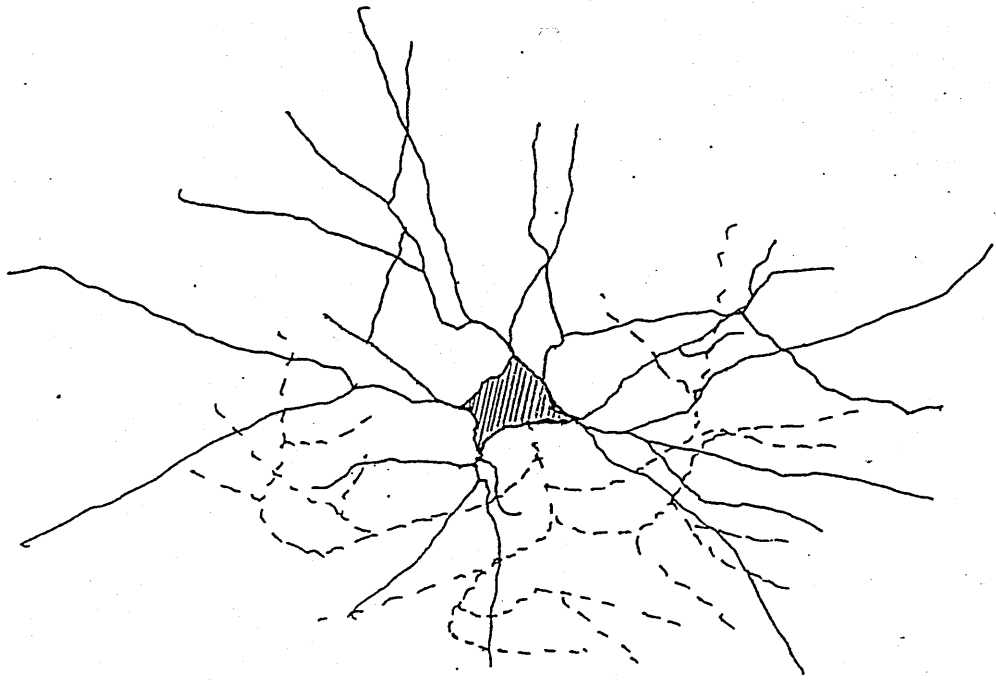


FIG. 12. A typical local circuit neuron, which has a maximum cell body diameter of approximately $16\mu\text{m}$. The axonal arbor is shown as dotted lines. Scale bar = $20\mu\text{m}$. See Tombol et al (1987) for a fuller description of the different types of local circuit and projection neurons in the IMHV.

because the dendrites are ordered from the cell body, where the first order dendrite is identified as the branch emanating from the soma (FIG.13). Therefore, even if some of the peripheral dendrite branches are truncated, the actual ordering of the entire tree will remain unchanged.

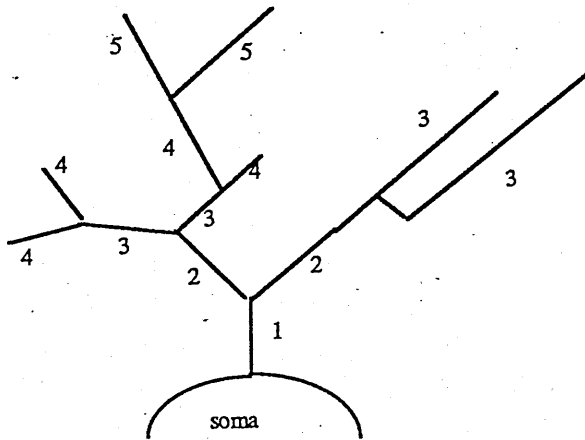
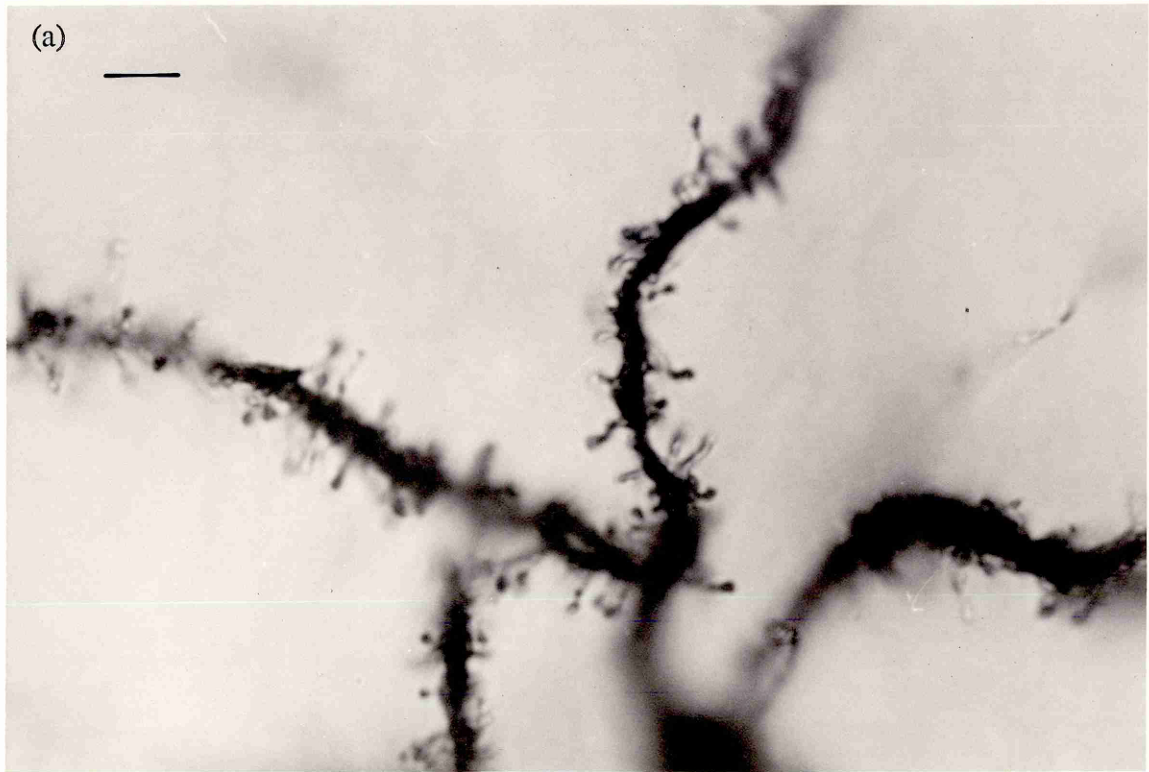


FIG. 13 Diagram showing the centrifugal ordering system used in the experiments reported in this thesis. The branches were ordered away from the cell body as shown.

In all, 52 cells from nine M- and 50 cells from eight W - chicks (1779 dendrite branches) were analysed. These 102 cells represent the maximum number of large multipolar, projection neurons that could be positively identified within the area studied. The dendrite length (DL), dendrite diameter (DD) and the overall spine lengths (SL) and spine head diameters (SHD) (FIG. -14) of all the visible spines along the dendrite branch were measured using an ocular graticule at a total magnification of x2000 on a Zeiss light microscope (photomicroscope III). The magnifications were calibrated precisely using an eyepiece and slide graticule. Any small protrusions from the dendrite shaft into the visible flanking zones of a length smaller than the average diameter of spine heads ($\sim 0.7\text{-}0.8\mu\text{m}$) that could not be identified unequivocally as a spine, were not included in the analysis - these



(b)

Abbreviations:
DD, diameter of dendrite
SL, length of spine
SIID, diameter of spine head
STL, spine stem length ($=SL-SIID$).

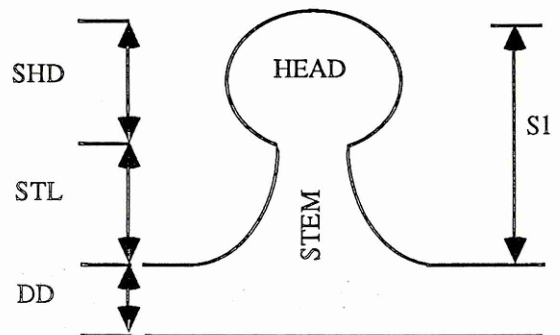


FIG. 14 (a) Photograph of a typical spiny dendrite from a large, multipolar, projection neuron (x2000). (b) Diagram of a dendritic spine showing measurements of spine length (SL) and spine head diameter (SHD) (the spine stem length (STL) = $SL - SHD$). The diameter and length of each dendrite branch was also recorded.

structures accounted for roughly 4% of the true number of spines. Also, dendrites that were obviously truncated, or obscured were not included in the measurements.

The spine densities (SD) were calculated as N/DL (no. of spines per μm of dendrite)(where N is the true total number of spines present on the dendrite, which was estimated using Feldman and Peters' correction formula). The *mean spine length*(SL), *mean spine head diameter*(SHD), *mean spine stem length* (STL=spine length minus spine head diameter), *dendrite diameter* (DD, averaged across the length of the dendrite branch) and *dendrite length*(DL) for each branch order was obtained.

3.4.5. STATISTICAL ANALYSES

The means of parameters SL, STL, SHD, SD, DD and DL, per dendrite order per hemisphere per chick, were analysed by ANOVA using an SPSS-X program (MANOVA) on a DEC-20 mainframe computer. A nested, repeated measures analysis of variance (ANOVA) (training by order within hemisphere) was adopted. Values for the first, sixth, and seventh order dendrites were excluded because the primary dendrites were short and thick with few spines and the latter two were infrequent: neurons from some of the brains did not have any visible sixth or seventh order dendrites and in some cases the dendrites left the plane of the section or they could not be measured. However, the means from the available data on these orders are still shown on the graphs to illustrate any trends that may exist. Following ANOVA, where two means were to be compared statistically, paired or unpaired Student's t-tests were used. Differences were considered to be significant at the 5%, or less, level.

3.5. RESULTS

The results are tabulated and presented in appendix A. The following therefore refers to the graphs which are shown here for the sake of clarity and conciseness. For a summary of the ANOVA results refer to the tables in appendix A.

SPINE DENSITY (SD) (FIG. 15). The spine density values are plotted against the dendrite branch order in FIG. 15, showing an increase in spine density of between 89 and 113% in the left hemisphere (FIG. 15a) and 37 to 69% in the right hemisphere (FIG. 15b) after training. The results of ANOVA give significant effects of training, hemisphere, and training by hemisphere (Table 1, appendix A), reflecting the significant increases in spine density after training and the significant hemispheric asymmetry present in W-chicks: the spine density in the right hemisphere is 47% greater, on average, than in the left hemisphere of W-chicks. This is significant for all orders (except the sixth) as given by paired t-tests (FIG. 15c). In the M-chicks however, this asymmetry is not present and the spine densities are approximately similar for all the dendrite orders, averaging 2.3 spines per μm for orders 2 to 5 (FIG. 15d).

There were also significant order, order within hemisphere, and training by order within hemisphere effects, but no training by order effects. This is shown in FIG. 15, where training affected the spine densities of different dendrites orders and differentially also in the two hemispheres.

DENDRITE DIAMETER (DD) (FIG. 16). Training did not significantly alter the dendrite diameters as shown by ANOVA (Table 2, appendix A). The only significant F-values obtained were for the order and order within hemisphere

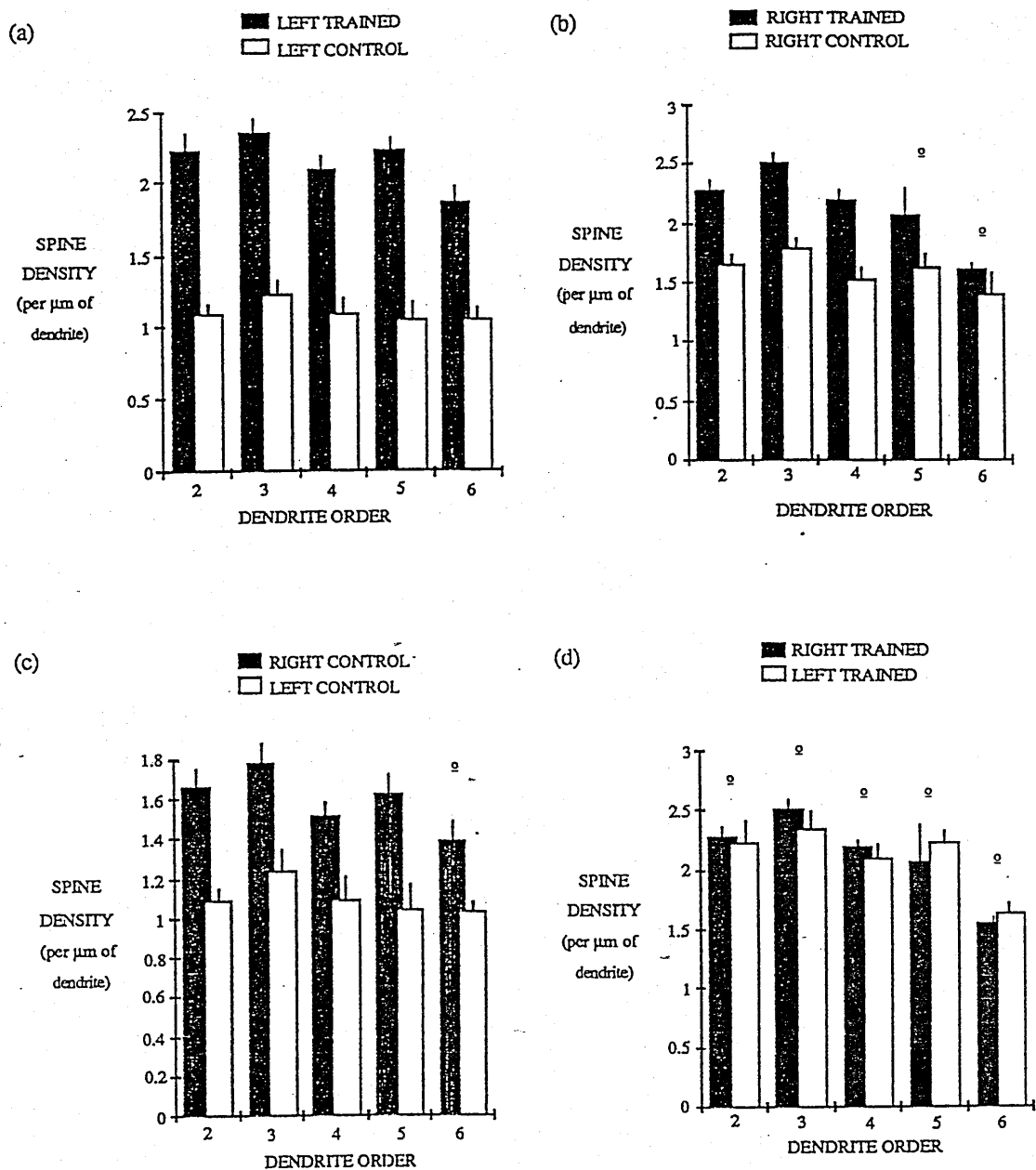


FIG.15 Spine density changes in multipolar projection neurons of the IMHV, 25h after training (a) in the left hemisphere and (b) in the right hemisphere; (c) shows a hemispheric asymmetry in control chicks that is abolished after training (d). Each bar is the mean spine density value from 9 trained (52 cells) and 8 control (50 cells) chicks and the vertical lines represent the standard error of the mean (SEM). All trained vs control differences are significant (by Student's t-test) at $p < 0.001$ for orders 2-5 except those indicated by ° = not significant. Only the 6th order of the left hemisphere shows significance at $p < 0.02$. MANOVA results are given in TABLE 1, appendix A.

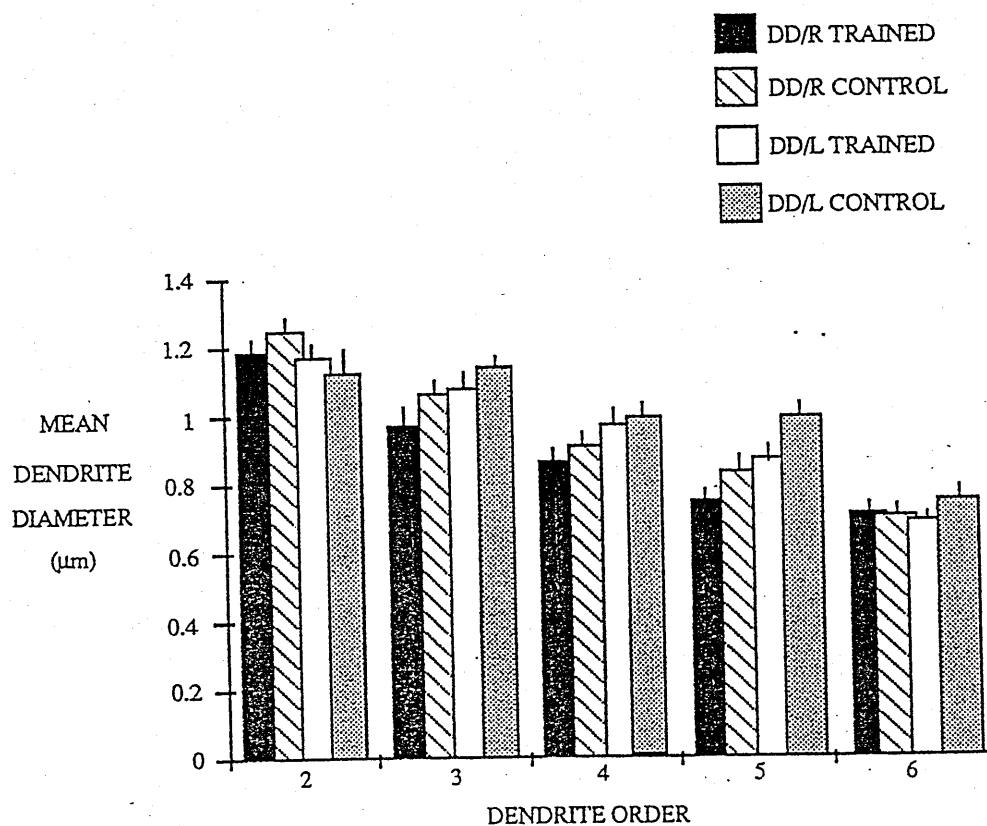


FIG. 16. Mean dendrite diameter (DD) differences after training: no significant training or overall hemisphere effects are found, although a significant order effect is present (see text). For the results of ANOVA see TABLE 2, appendix A. R=right hemisphere, L=left hemisphere.

effects, which reflects the thinning of the dendrite branches as the dendrite order increases. This is mainly due to the fact that the lower order branches bifurcate to give two higher order dendrites (FIG. 16).

DENDRITE LENGTH (DL) (FIG. 17). Total dendrite length per neuron was not measured because most of the neurons were either truncated or obscured by other processes. However, a mean dendrite length for each branch order was calculated (FIG. 17) which alters - either increasing or decreasing - according to the branch order and the hemisphere. Although none of the trained vs control differences were significant, paired t-tests revealed significant hemispheric asymmetries only in orders 2 and 3 in control chicks: the left is significantly greater than the right. This is also shown by ANOVA (Table 3, appendix A) which gives significant hemisphere, order, and order within hemisphere effects only.

SPINE LENGTH: (SL) (FIG. 18) There was no statistically significant effect of training on the average spine length for any of the dendrite orders in either hemisphere, although there is an apparent reduction of 3.3 to 6% in most of the dendrite orders (FIG. 18). The 0.8 to 3.4% increase in spine length in dendrite orders 4 and 5 of the right hemisphere and order 5 of the left hemisphere is due mainly to a large increase in spine head diameter. This, as FIGs 19 and 20 show, occurs without a significant shortening of the spine stem. Table 4 (appendix A) shows the ANOVA results which only gives a significant order within hemisphere effect.

SPINE HEAD DIAMETER: (SHD) (FIG. 19). By ANOVA: there were statistically significant effects of training, training by hemisphere, order within hemisphere, and

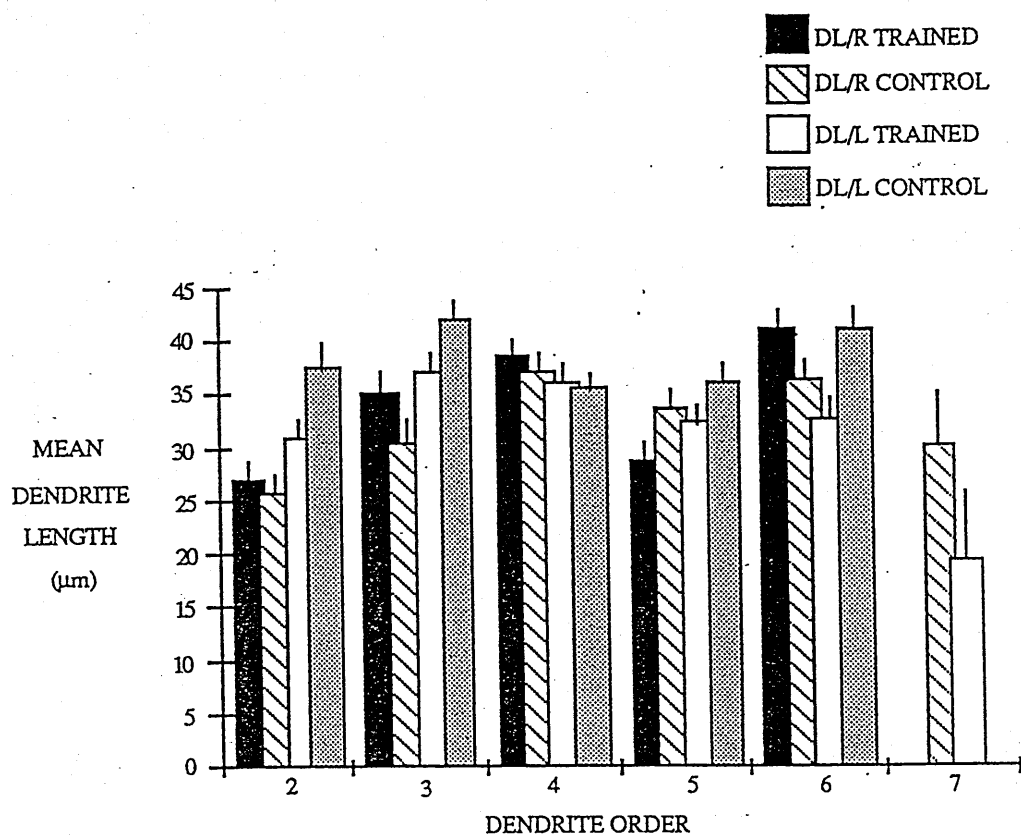


FIG. 17. Mean dendrite length (DL) changes after training. No significant training effects are present, although there is a hemispheric asymmetry in control chicks in the 2nd ($t(7)=3.091$, $p=0.011$) and 3rd ($t(7) = 3.438$, $p = 0.017$) orders. ANOVA results are given in TABLE 3, appendix A. R=right hemisphere, L=left hemisphere.

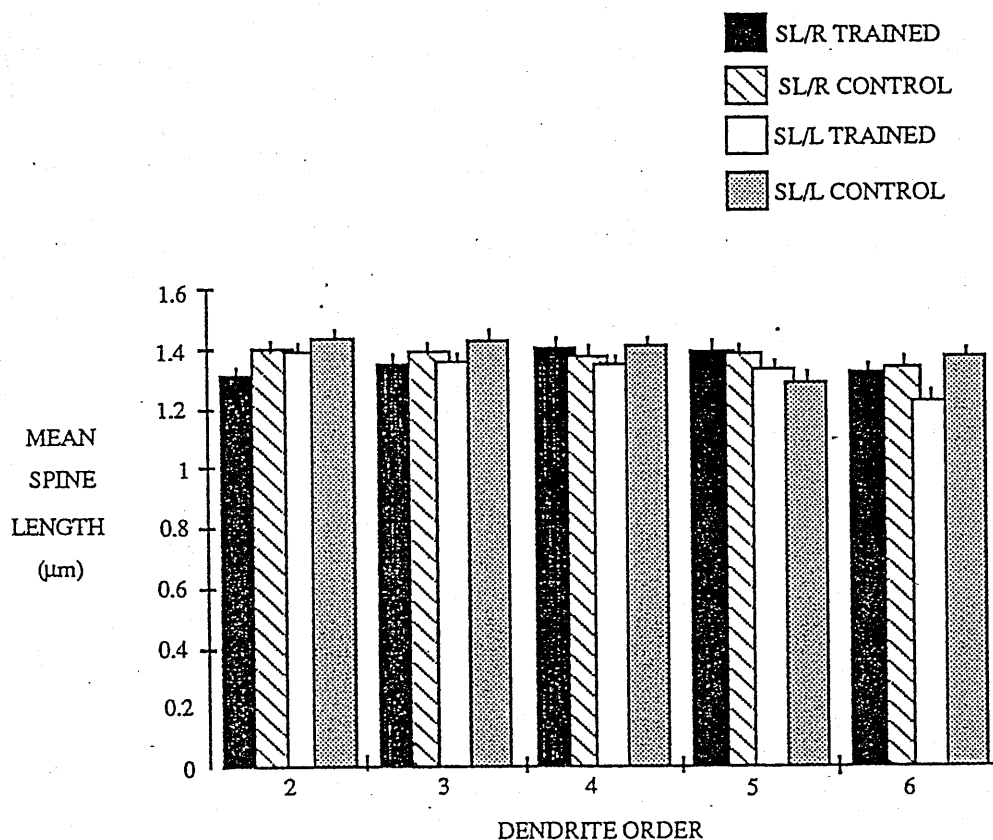


FIG.18. Mean spine length (SL) changes in the various branch orders of multipolar projection neurons 25h after training: no significant training effects are present. ANOVA results are presented in TABLE 4 appendix A. R=right hemisphere, L= left hemisphere.

training by order within hemisphere effects, but no hemisphere effect (Table 5, appendix A). In the left hemisphere, training significantly increased the mean spine head diameter of orders 2,3 and 5 by between 5.3 and 8.8%, as determined by t-tests (FIG. 19a). There was no significant increase in spine head diameter in the right hemisphere (FIG. 19b). The significant interaction terms reflect the apparent reversal of hemispheric asymmetry after training: the mean spine head diameter in the right control hemisphere was some 0.5 to 4.4% greater than the left (FIG. 19c), whereas after training the spine head diameter in the left trained hemisphere was between 0.2 to 3% greater than the right (FIG. 19d).

SPINE STEM LENGTH: (STL) (FIG. 20) The large increases in the spine head diameter with relatively unaltered spine lengths indicates that the overall spine stem length was shortened in M-birds. ANOVA gives a significant training, order within hemisphere and training by order within hemisphere effects only (Table 6, appendix A). These results reflect a decrease of between 2.5 and 16.8% in the left hemisphere in orders 3,4 and 6, which is statistically significant (FIG. 20a) and between 1.3 and 13.6% in the right hemisphere (not significant) (FIG. 20b). Although the hemispheric differences in spine stem length between the control birds were not significant, the left control hemisphere had spine stem lengths between 4.9 and 9.4% longer than the right. Training reversed these asymmetries, although significantly only in orders 4 and 5, with the right trained hemisphere having longer spine stem lengths than the left, by 1.1 to 11.5% (FIG. 20c,d) (although all orders are affected, these changes seem to occur mainly in the middle and distal dendrite orders).

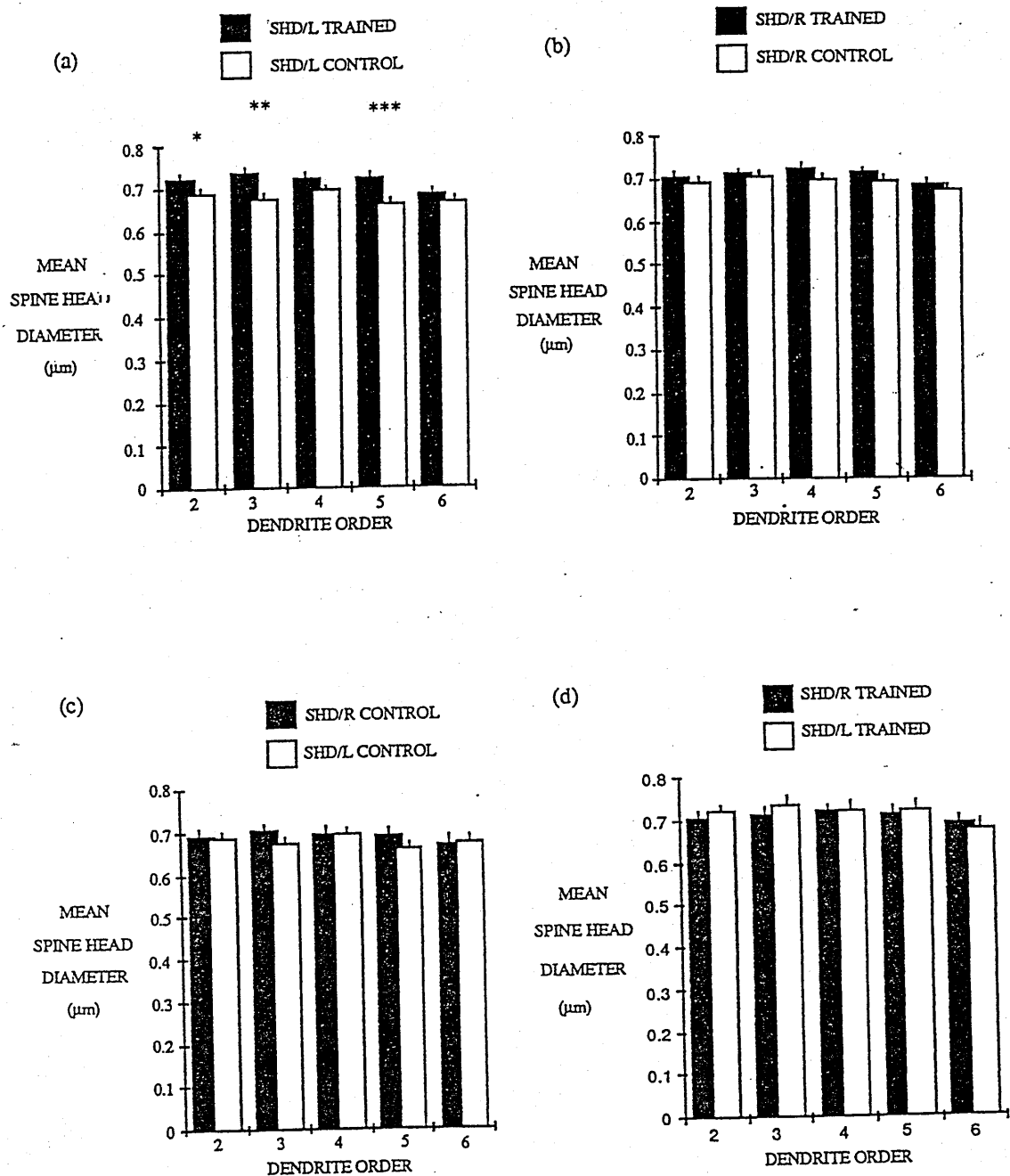


FIG.19. Mean spine head diameter (SHD) changes 25h after training. Only the left hemisphere (a) shows significant increases in the spine head diameter in orders 2 ($t(15)=2.132$, $*p=0.05$), 3 ($t(15)=2.179$, $**p<0.02$) and 5 ($t(15)=4.828$, $***p<0.001$). (b) The small increases in the right hemisphere are not significant. No hemispheric asymmetries (c, controls and d, trained) are present. The ANOVA results are given in TABLE 5, appendix A.

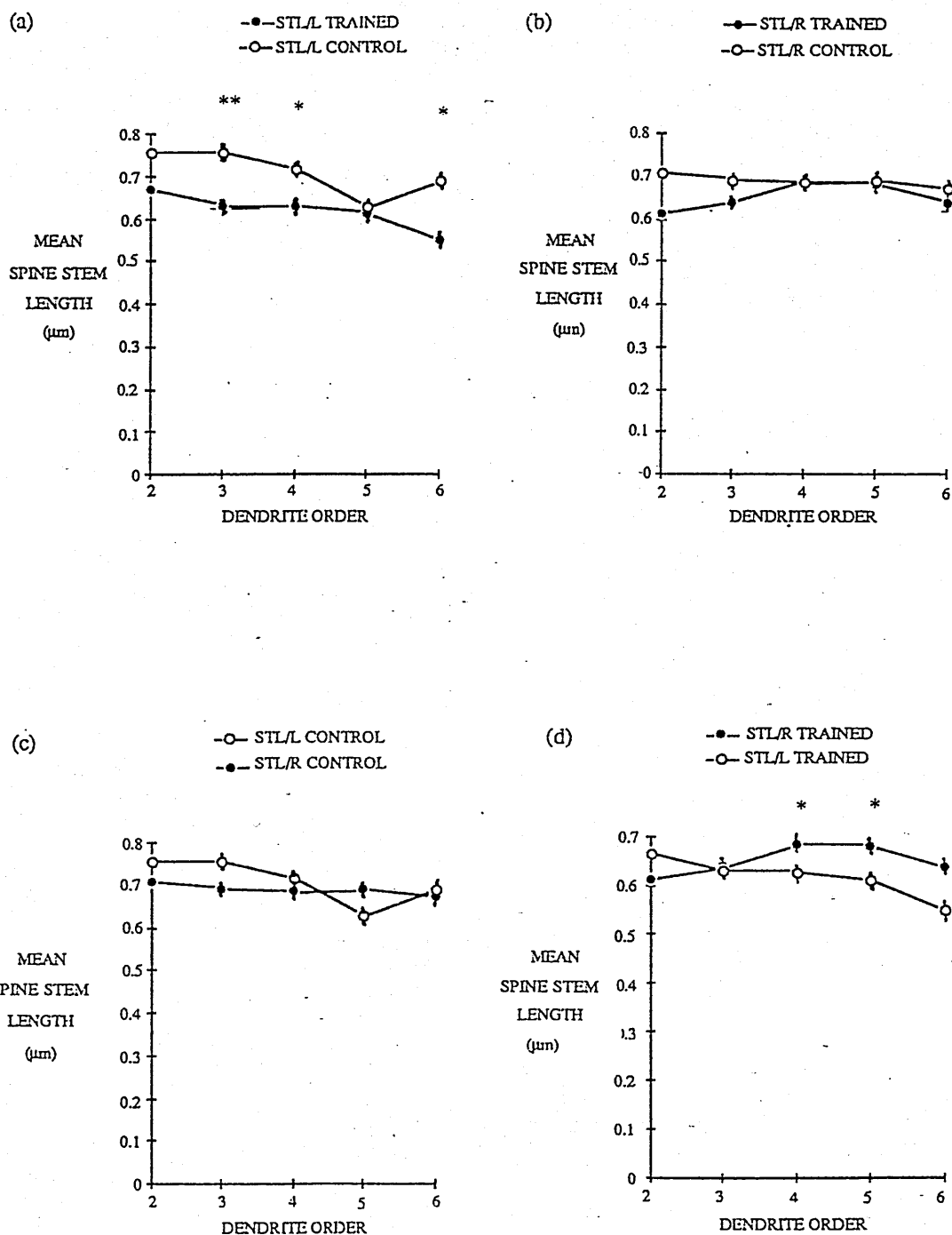


FIG.20. Mean spine stem length (STL) changes 25h after training. Again, Only the left hemisphere shows significant training effects (a): The STL is shorter in orders 3($t(15) = -3.45$, $**p < 0.02$), 4: $t(15) = -2.397$, $*p < 0.05$) and 6($t(9) = -2.405$, $*p < 0.05$). No significant changes are found in the right hemisphere (b) and there is no hemispheric asymmetry in control chicks (c). However, because only the left hemisphere shows increases in spine head diameter and decreases in STL, a hemispheric asymmetry in trained chicks (d) is to be expected, although only significantly in the 4th($t(8) = 2.848$, $*p < 0.03$) and 5th($t(8) = 2.429$, $*p = 0.04$) orders. ANOVA results are presented in TABLE 6, appendix A.

3.6. DISCUSSION / EXPERIMENT.1

The aim of the first series of experiments was to determine the effects of passive avoidance training on dendritic spine parameters of multipolar, projection neurons in the chick IMHV. The other cell type present - local circuit neurons (FIG. 12) (Tombol et al, 1987) - did not show (as mentioned earlier) any qualitative change in spine number and were therefore not analysed. An important point to bear in mind is that not all parts of the dendrites of each multipolar neuron examined will have been measured because sectioning the tissue into 90-120µm thick sections will undoubtedly remove many of the dendrites, or parts of dendrites on neurons in which the diameter of the dendritic field is approximately 200-250µm.

However, this caveat notwithstanding, the above investigation has shown changes in the number and shape of dendritic spines on LMPNs 24-26h after passive avoidance training. Interestingly, some hemispheric asymmetries have also been found: significant changes in most of the parameters examined have been observed mainly in the left hemisphere after training. These include a maximum *increase* of 113% (orders: 5th>2nd>4th>3rd>6th) in spine density (FIG. 15); up to a 20% *decrease* in spine stem length, in the 3rd, 4th and 6th order dendrites (FIG. 20), and a 9% *increase* in mean spine head diameter, but only in orders 2, 3 and 5 (FIG. 19). The mean spine length did not change significantly after training in either hemisphere, which suggests that the observed spine shape changes in the left hemisphere are due mainly to three possible causes: (1) a significant elongation of the spine head alone - as in the 2nd and 5th order dendrites, or (2) a significant spine stem length shortening alone - as in the 4th and 6th orders, or (3) both spine stem shortening and head elongation - as in the 3rd order dendrites.

In contrast to the numerous alterations occurring in the left hemisphere after training, there were no significant differences in any of the spine shape parameters examined in the right hemisphere, although a significant increase in spine density was present in orders 2, 3 and 4 (FIG. 15b). In the right hemisphere, as in the left, there were no obvious spine length changes following training (FIG. 18). These differences between the hemispheres are perhaps a function of the hemispheric asymmetries in W-chicks. The parameter values (spine density, spine head diameter, and spine stem length) for the right hemisphere are such that they are already greater in the control animals, so that any increases occurring as a result of training are not sufficiently large to make the differences significant. This is the case for the spine shape changes: the reason why the slight increases in spine head diameter in the right hemisphere after training are not significant, is because the values are already greater in the controls (FIG. 19). In comparison, the values in the left control hemisphere are sufficiently low so that the increases in spine head diameter occurring as a result of training, become significant. A similar argument can be proposed to explain the significant changes in spine stem length found only in the left hemisphere after training. This is also partially true for the spine density changes, where there is a significant hemispheric asymmetry in the controls with the values in the right being greater than those in the left. After training, the spine number significantly increases in both hemispheres, but to a lesser extent in the right hemisphere, such that the asymmetry is subsequently abolished (see FIG. 15).

One of the factors which may influence these large increases in spine density is dendrite length: the dendrite may be 'compressed', thereby giving the appearance of a greater spine density. Such an effect, however, is unlikely because as FIG. 17 shows, there were no significant changes in mean dendrite length in any of the branch orders, and the branch lengths actually increased in the lower order

dendrites of the right hemisphere where significant increases in spine density were found.

It could also be argued that these morphological alterations are the result of non-specific effects of any concomitants of the training experience, such as arousal, the release of stress-related hormones and the perception of the taste of the methylantranilate. If hormones are the cause of these changes, then they would be expected to have a generalised effect on the morphology of the entire neuron. Therefore, gross changes in the branching patterns of the LMPNs should also be observed in the trained birds. This study is reported in chapter 5.

To test for the effects of these non-specific factors, further experiments need to be performed, and one way of accomplishing this objective is to study the morphology of LMPNs in the IMHV of trained chicks which are experimentally rendered amnesic, compared to those which can still remember. This would control for any of the non-specific effects of the concomitants of the training experience.

CHAPTER 4

EXPERIMENT 2: The specificity of the spine density and shape changes to memory formation.

4.1. INTRODUCTION.

One method of inducing amnesia in trained chicks is by administering a brief subconvulsive, transcranial, electroshock through external electrodes placed on either side of the head. Amnesia will be produced if the shock is given immediately after training, in that the chicks will subsequently peck at the dry test bead (Rose and Harding, 1984). By contrast, if the shock is delayed to ten minutes after training, the trained chicks will show recall and avoid the test bead. This procedure abolishes the training related increases in high frequency neuronal firing in the IMHV (Mason and Rose, 1988) and the increase in ^3H -fucose incorporation into the forebrain (Rose and Harding, 1984) of those chicks which had been trained but subsequently rendered amnesic as a result of the electroshock. Thus we can conclude that these electrophysiological and biochemical changes are not due to concomitants of the training procedure, such as the perception of the taste of the methylanthranilate, but are likely to be associated directly with the process of memory formation.

Rose has shown that by altering the time of administration of the shock relative to the training experience, it is possible to alter the proportion of trained birds which show recall to those that forget the task (i.e. those rendered amnesic). Thus, if a population of trained birds is shocked five minutes after training, then about half will show recall and half are amnesic on test. Chicks trained on a water-coated bead and subsequently shocked in a similar way do not show any changes either in

pecking behaviour, or on the biochemical and physiological measures so far studied (Rose and Harding, 1984; Mason and Rose, 1988).

Thus, using this electroshock treatment, it is possible to generate two groups of birds, both trained identically and tasting the bitter bead, but only one of which is amnesic, the other showing recall. The resulting biochemical and electrophysiological differences between these two groups are suggested to reflect memory storage processes rather than any other training-related concomitants. Therefore, the aim of this third study was to utilize this electroshock paradigm to induce amnesia in trained chicks, and to determine whether the morphological changes in dendritic spines were specific to memory formation processes.

4.2. METHOD.

M- and W- chicks trained exactly as described previously (chapter 3) were therefore given a brief, subconvulsive, transcranial electroshock (12mA, 110v, 220ms duration at 50Hz), 5 min after training. Three groups of birds were obtained, as a result:

- (1) shocked M-chicks which show recall for the task (i.e. avoided the dry bead on subsequent testing 30 min and 24h after training),
- (2) shocked M-chicks which were rendered amnesic (i.e. pecked the dry bead on both tests), and
- (3) shocked W-chicks which still pecked the dry test bead.

If the changes in dendritic parameters previously observed were a consequence of the experience of tasting methylantranilate compared to water, then these

morphological changes should be apparent in both the recall and amnesic groups (1 and 2) by comparison with the water control group (3). If, on the other hand, the dendritic modifications are associated with memory storage rather than the mere experience of methylanthranilate, then the changes should be present in the shocked recall group (1), but not in the amnesic group (2), by comparison with the water group (3), and there should be significant differences on these measures between the recall and amnesic groups (1 and 2).

The Golgi, methods and measurements were exactly as described in chapter 3, except that here there were seven chicks in each of the three groups (amnesic, recall and water). Steven Rose, of the Brain and Behaviour Research Group, performed the training and administered the electroshock because only he held a license to carry out this procedure. In each replication of the experiment, one chick from each of the three groups was perfused and Golgi impregnated by me as described previously. The chicks were coded so that all of the perfusions and the measurements were performed "blind"; the codes were only broken after the data had been collected.

Three to four large, multipolar, projection neurons were analysed from the IMHV regions of the left and right hemispheres of each of the seven chicks from each of the three groups. Only the 3rd order branches were further analysed in this experiment because the previous study demonstrated that only 3rd order branches showed significant changes in all three parameters: an increase in spine density, with spine head diameter enlargement and spine stem length shortening. Other branch orders showed only one or the other of the spine shape changes, together with a concomitant spine density increase (see chapter 3). The same measures were made: the spine head diameter (SHD), overall spine length (SL), dendrite length

(DL), dendrite diameter (DD) and mean spine stem length (STL) for each dendrite branch (=SL-SHD). The correction formula of Feldman and Peters (1979) was applied to the data to obtain an estimate of the true total number of spines (N) present on the dendrite, and the spine density (SD) calculated as N/DL (number of spines per μm of dendrite). The mean values for each of the six parameters (SHD, SL, STL, DD, DL, SD) per hemisphere per chick were then analysed by a repeated measures ANOVA using an SPSSX package. For significance testing between two groups, a one-tailed student's t-test was applied, because the experiment was designed to test specific predictions about the direction of any changes.

4.3 RESULTS and DISCUSSION / EXPERIMENT. 2

Spine density significantly increased in chicks showing recall of the training task, compared to those either rendered amnesic or to the shocked water controls (FIG. 21) (By ANOVA: $F(2, 18)=3.29$, $p<=0.06$). This increase of 28% was lateralised to the left hemisphere ($t(12)=2.071$, $p=0.029$, compared to the amnesic group; $t(12)=2.018$, $p=0.032$, compared to the water group); the 15% increase in the right hemisphere was not significant ($t(12)=1.28$, $p=0.11$, compared to the amnesic group; $t(12)=1.399$, $p=0.093$, compared to the water group). These results support the argument that the increase in spine density after passive avoidance training is specifically related to memory formation processes, because the chicks which were rendered amnesic did not show any change in spine density compared to the shocked water controls.

There were no significant changes in dendrite length or dendrite diameter between chicks in the three groups (Table 7, appendix A). The significant spine shape changes (increases in spine head diameter and spine stem length shortening) found

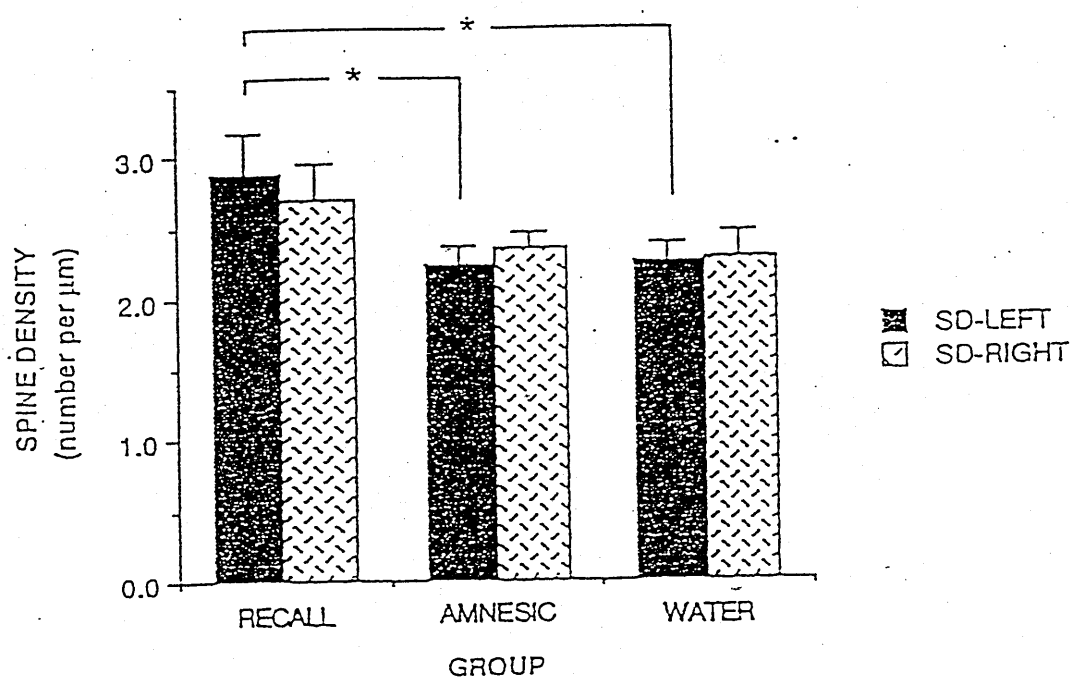


FIG. 21. Spine density changes in electroshocked chicks (\pm SEMs). Only those chicks that recalled the task showed an increase in spine density compared to those which were rendered amnesic and to the shocked water controls; $n=7$ in each group. Significant changes were observed only in the left hemisphere. ANOVA results are given in TABLE 7, appendix A.

between unshocked M- and W- birds in the previous study 25h after passive avoidance training were not observed here (Table 7). There were no significant differences in spine shape in trained chicks which were shocked, but still showed recall of the task, compared to chicks rendered amnesic by the electroshock. This suggests that changes in spine shape may not be connected with engram formation *per se*, but are possibly a result of physiological factors relating to the training experience itself. It could also be argued that the electroshock may have had a disruptive effect on the structure of the spines, over and above those changes due to memory formation processes. Such a result is possible because dendritic spines are very plastic structures, whose shape can change rapidly (within 4min) upon intense electrical stimulation (Fifkova and van Harreveld, 1977; Fifkova et al, 1982). However, before such a conclusion can be reached, the effect of the electroshock *per se* on spine dimensions in the resting brain has to be established.

Because the overall spine length did not change after passive avoidance training in either this study, or in the earlier spine morphology experiment, the increase in spine density cannot be fully explained by supposing that more spines that were previously hidden or just noticeable from behind and in front of the opaque Golgi-impregnated dendrite shaft, became visible after training and were therefore counted in the analysis. For this to occur all of the spines should lengthen, and not just those that were previously invisible. Also, there was no correlation between the *visible spine density and spine length* for grouped raw data (see table 10, appendix A). The only significant correlation found was that between the *visible spine density and the corresponding dendrite diameter* only for the amnesic group (Table 10), and contrary to expectation this was highly *positive*. That is, the thicker the dendrite diameter, the greater the visible spine density. One would expect the

opposite: thinner dendrites should show more spines projecting from the opaque dendrite shaft and hence bias the resulting spine count. However, the combined effect of a slight lengthening of the spine, the enlargement of the spine head and the thinning of the dendrite shaft which occurs after training may account for a small proportion of the increase in spine density. Also, because both the dendrite lengths and the dendrite diameters have changed after training, the dendritic membrane may have been redistributed into forming new spines. To account for the large increases in spine density, it seems most probable that new membrane and cytoskeletal proteins are synthesised for the production of new spines, within the 24-26h period following the training experience. The implications of these results will be discussed further in chapter 6.

Additional questions may be asked regarding the extent of the plastic change occurring as a direct consequence of the passive avoidance training: (1) Are there also alterations in the branching patterns of dendrites? (2) Can the branching patterns of the LMPNs alter within 25h of a single-trial learning experience?

CHAPTER 5

EXPERIMENT 3: Dendritic branching changes 25h after training.

5.1 INTRODUCTION.

Dendritic branches receive the greatest proportion of synaptic contacts on a neuron in comparison to those on the soma (95-99%; Rall and Segev, 1987), and therefore are the major sites at which information processing occurs in a given neuron. The amount of information processing occurring on the neuron is perhaps related to the complexity of the branching, because all of the inputs on a dendritic tree tend to be summated at the primary dendrite, and the soma. Horwitz (1981) has provided some evidence on the processing capabilities of dendritic branching. If greater branching occurs in the region peripheral to the input more current will be drawn to the periphery, so that less will be received at the soma. As a result, the peak voltage at the soma will be reduced. Thus, changes in dendritic branching patterns of neurons (including the density of spines present on the branches) will reflect changes in the amount of information that a particular neuron can receive and subsequently process. Such changes may be particularly relevant in information storage processes, where new synapses form and dendritic branches may extend or form *de novo* to make connections with other axons. Indeed, the plasticity of dendrites has been studied *in situ* by Purves and Hadley (1985) who visualised the same neuron in the superior cervical ganglion of young adult mice at intervals of up to 33 days. They found alterations in dendritic branching *in vivo* within a two week period: some branches retracted, some elongated whilst others seemed to form *de novo*.

Many of the early experiments on changes in dendritic branching were primarily performed in rats subjected to "enriched" experience for up to 30 days from the time of weaning (EC rats). These were compared to rats reared in isolation (IC rats) or simply with conspecifics in cages (SC rats). The EC rats had 15% more dendritic material in pyramidal neurons of layers II, IV, and V, as well as layer IV stellate cells in the visual cortex compared to isolates and group housed rats (Volkmar and Greenough, 1972; Greenough and Volkmar, 1973; Holloway, 1966). Granule cell dendritic trees of the hippocampus were also more highly branched in the EC compared with IC or SC rats (Fiala, 1978). Similar changes in dendritic branching patterns have also been found in adult rats, although to a lesser degree, in the environmental enrichment paradigm (Green et al, 1983). The initial experiments on EC versus IC rats (pre 1981) were based on measurements using the Scholl (1956) concentric ring method (fig.17), which relies on camera lucida tracings of the relevant neurons, and therefore has several drawbacks which are discussed later.

The EC rats also showed gross morphological alterations in various regions of the rat cortex, particularly the visual cortex, which was heavier and thicker, and had larger nerve cell bodies and more glial cells (Diamond, 1967; Diamond et al, 1966; Sirevaag and Greenough, 1985). The spine density was also greater on pyramidal neurons of the visual cortex in EC rats (Globus et al, 1973), together with a larger number of synapses per neuron (Turner and Greenough, 1985).

Whilst the above effects on dendritic branching were primarily the result of increased, non-specific sensory experience, later studies have looked at changes associated with learning experiences, particularly maze-training and more recently operant conditioning (Mahajan and Desiraju, 1988). For example, in young adult rats, 30 days of maze-training causes increases in upper apical dendritic fields in

both layer IV and V pyramidal neurons in the visual cortex, compared to handled controls (Greenough et al, 1979). To test for the effects of general factors related to the training procedure such as stress or motor activity, these experiments were repeated in split-brain rats. One eye was occluded throughout the experiment during the maze training sessions, thereby providing a within subjects control (Chang and Greenough, 1982). The hemisphere opposite the open eye of trained rats had larger upper apical pyramidal dendritic fields in the occipital cortex (measured using a computer microscope), suggesting that the morphological changes are due to visual input from the training experience rather than as a consequence of general hormonal or metabolic effects of the training experience. Similar results have also been found for a reach training paradigm in which adult rats were trained to reach for food with either their preferred forepaw or their nonpreferred one, with subsequent hemispheric comparisons, showing more second order branches in the hemisphere opposite the trained forelimb (Greenough et al, 1985b). Seven days of operant conditioning of rat pups also changes dendritic branching and increases spine densities of hippocampal CA3 pyramidal neurons (Mahajan and Desiraju, 1988).

Perhaps the best evidence for the involvement of dendritic branching in the processing and storing of learnt information comes from studies of song acquisition in various species of bird: canaries, zebra finches, and sparrows. The song control nuclei in these birds, in particular the robustus archistriatalis (RA), are several times larger in males than in females. This correlates well with the fact that only males of these species sing (Nottebohm and Arnold, 1976; Gurney and Konishi, 1980; DeVoogd and Nottebohm, 1981a). The dendritic fields of neurons (large, spiny, multipolar with 3-5 primary dendrites) from the RA of males were found to be larger, projecting some 22% further from the cell body, than those in females

(DeVoogd and Nottebohm, 1981a). Even larger differences between the sexes were observed in zebra finches (DeVoogd et al, 1986). That these alterations may be causally related to the encoding, or the potential for encoding, the song that is acquired from conspecifics, is partly demonstrated by the observation that in those species of birds where both sexes sing, large dendritic branching differences are absent, together with minimal differences in the sizes of the song control nuclei (DeVoogd et al, 1988).

However, data is lacking regarding the rapidity of the changes in dendritic architecture and to its specificity in memory formation processes. That is, given that memory storage, which may involve the formation of new synapses, can occur within 24 to 48h of learning, can dendrites alter their branching patterns within this time period? From the above experiments, it is known that changes in spine density are possible in a 24h time period, and that these are specifically related to memory formation processes (see chapter 4 above), but is this also true of dendritic branching? Can the dendritic trees of large, multipolar, projection neurons alter after a *single* specific learning experience, which in this case is a one-trial passive avoidance learning task?

Answers to these question will provide some information regarding the extent of the plastic response in memory formation for passive avoidance training and its generalisation for other learning tasks and across species. Also, because the above studies have found an increase in spine density after training, any redistribution of the membrane likely to have occurred as a result of these changes, may manifest itself in a corresponding change in dendritic branching patterns of the LMPNs.

A study of dendritic branching patterns, particularly on multipolar neurons whose

arbors sometimes extend up to 250µm, means that several methodological problems need to be solved. The primary one is that of tissue sectioning, which will truncate part of the dendritic tree, and thus give an underestimation of the true extent of the dendritic field of neurons. Also, dendritic branches are rarely arranged in one focal plane; they invariably follow a course through the thickness of the section at an oblique angle. To overcome such obstacles, several investigators have developed computerised microscopes that can allow the experimenter to follow the dendritic branches at different focal depths and from one section to the next, thereby giving a three dimensional view of entire dendritic fields of neurons (DeVoogd and Nottebohm, 1981a; DeVoogd et al, 1981).

However, such technological aids were in the process of being developed in the Open University, and were therefore, unfortunately, not available for the present study. Earlier dendritic branching studies used the camera lucida approach in which the three dimensional (3D) structure of dendritic trees was projected into two dimensions (2D). Significant differences were found between the experimental and control groups (e.g. Volkmar and Greenough, 1972; Greenough and Volkmar, 1973; Holloway, 1966; Fiala et al, 1978; Greenough et al, 1979; Mahajan and desiraju, 1988). It was therefore decided to use this method to study the branching of LMPNs drawn by camera lucida from the IMHV regions of trained and control chicks.

5.2 METHODS.

In order to investigate alterations in dendritic branching in the passive avoidance training paradigm, 25h later, some of the slides of the sections taken from the IMHV regions used for the first spine density study (chapter 3) were therefore re-

analysed for dendritic branching changes. These slides represented only 4 trained and 4 control chicks from the previous study, because of problems with the section mounting medium (DPX), which shrank and therefore allowed some of the sections to dry out. The useable slides were re-coded so that experimenter bias could be avoided. More chicks were trained and perfused giving a total of 8 trained and 8 control chicks, which were also coded. The subsequent perfusion, dissection and Golgi procedures were exactly as described previously (see expt. 1, chapter. 3).

Identified LMPN neurons were drawn with the camera lucida attachment of a Zeiss light microscope at x500 (FIGS. 11 and 22). 52 neurons from the left and right IMHV regions from trained chicks and 51 neurons from controls were drawn. These were analysed in three ways; (1) in terms of the numbers of branches of each order of dendrite per dendritic tree, which will provide information concerning the probability of any new branches having been formed after training, (2) by a modified Sholl (1956) concentric ring analysis method, which will indicate branching complexity and changes in the extent of the dendritic field as a result of training and (3) a topological analysis, based on the vertex analysis method devised by Berry et al (1986), which involves analysing the frequencies of various distinct branching patterns. This will give an indication of changes in the pattern of branching as a result of training.

This latter analysis was performed in order to partly overcome some of the criticisms (Uylings et al, 1986) levelled at the Sholl (1956) method of concentric ring analysis, i.e. that this method alone does not provide any information regarding (1) the *topological* size and pattern of the individual dendritic trees, (2) the metric length of the different segments, and (3) the branching angles in each dendrite of the neuron. The measurements of the metric lengths of the individual

segments and the branching angles would have to await a 3D analysis of the actual neurons using a computer-aided microscope.

NUMBER OF BRANCHES: To determine the number of branches, the individual dendritic trees of each neuron were redrawn schematically (as in FIGs. 13 and 23) so that ordering of the dendrites, by the centrifugal method, could be accomplished with ease. The numbers of branches of each order away from the cell body were then totalled for each dendritic tree and then averaged for all of the trees on a given neuron for each branch order. This gave the mean numbers of branches for each order of dendrite per dendritic tree, for each neuron.

The means of these values for each chick were then calculated and statistically analysed using ANOVA on the SPSSX package (MANOVA program) on the mainframe computer (Training by order within hemisphere; hemisphere being a within subjects factor).

SHOLL ANALYSIS: Twelve concentric circles, 15 μ m apart, were drawn on a piece of tracing paper so that the same sheet was used for the analysis of all of the neurons (which were all drawn at the same magnification (x500) and at the same camera lucida setting. Also a scale bar was added on each of the neuron drawings for comparison purposes). The tracing paper was placed on the drawing of the neuron, and the focal centre of the concentric rings aligned with the centre of the cell body by eye (FIG. 22). The paper was placed on a light box so that the illumination from below allowed easy measurements of the numbers of intersections by the dendrites with the concentric circles (rings).

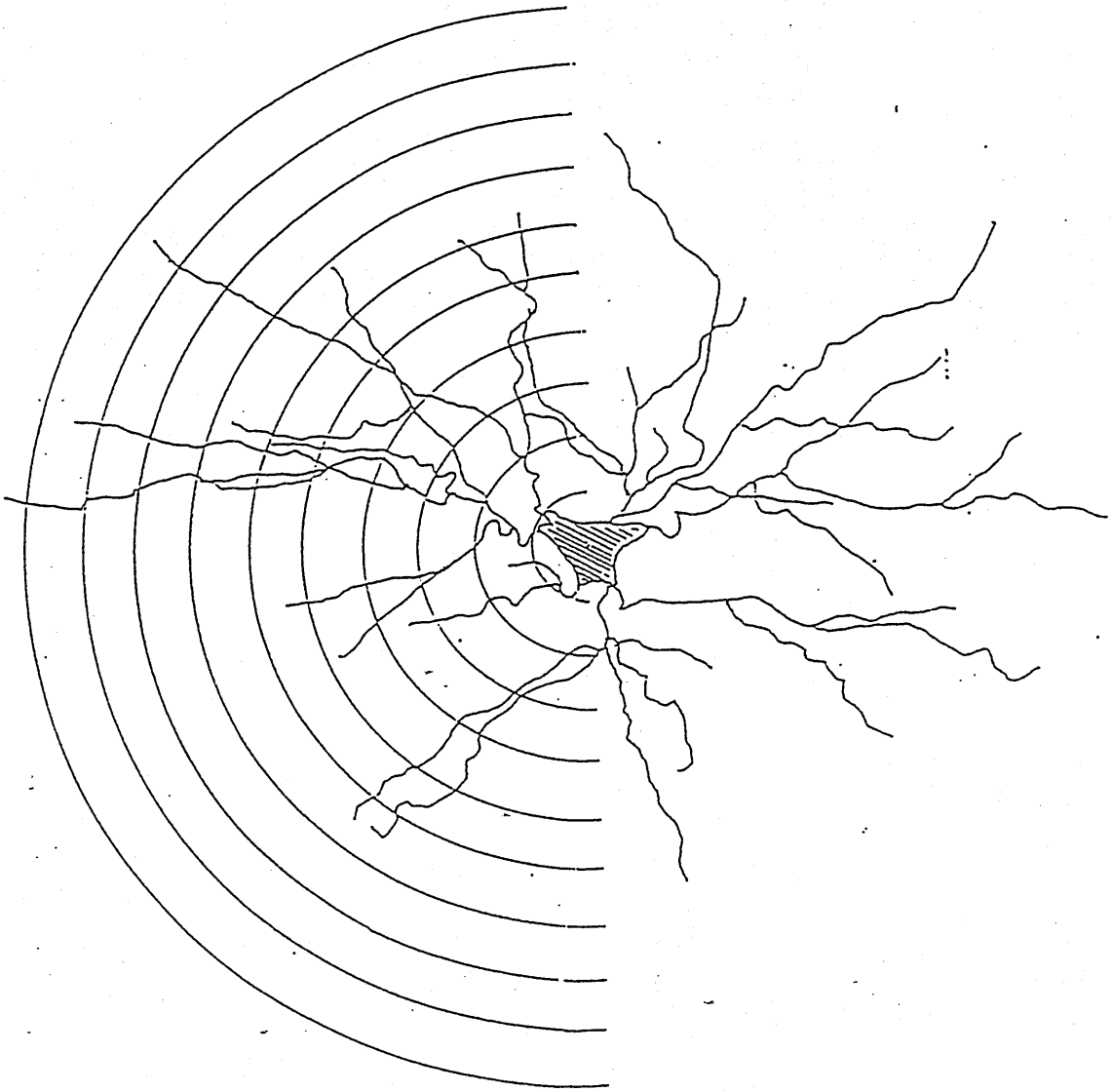


FIG. 22. The sholl analysis of dendritic branching complexity. The concentric circles (half of which are shown above for illustration purposes) were drawn on a piece of tracing paper and laid on top of the camera lucida drawing of a large, multipolar projection neuron (not showing the axon). 10 such semicircular rings are shown above. Each dendritic tree was followed from the cell body and the number of intersections with the rings (numbered from the cell body) with the dendrite branches was counted. The number of ring intersections per ring per tree per neuron was calculated for each neuron analysed. (see text).

The values were standardised to a mean number of ring intersections for each ring per dendritic tree of the neuron. Data was then statically analysed by ANOVA as described previously, using mean values of the number of intersections for each ring, per tree, for each chick. However, because the ANOVA program could not handle more than five levels of each factor, groups of rings were analysed separately between trained and controls: rings 1 to 4, 5 to 8, and 9 to 11 (see Table 9, appendix A).

TOPOLOGICAL ANALYSIS The topological method used here was based on Vertex analysis [designed by Berry and colleagues (Berry et al, 1986; Sadler and Berry, 1983; Berry and Flinn, 1984)]. The terminology used in vertex analysis is summarised in FIG. 23, and is based on the centrifugal ordering method (FIG. 13). Vertices - which can be terminal, root and link - are points in a network that are connected by segments. "Dichotomous vertices" are branches giving rise to two other dendritic segments (arcs) which can either be links or terminals. Hence, three possible types of dichotomous vertices are possible: Va, which has two terminal segments; Vb, which has 1 link and 1 terminal segment; and Vc, which has two link segments. Similarly, dendrites which give rise to three other segments are termed "trichotomous vertices", which have four possible combinations of terminal and link arcs which are labelled V-ta (3 terminal arcs), V-tb (2 terminals and 1 link), V-tc (1 terminal and 2 links), and V-td (3 links) in FIG. 23.

The mathematical interrelationships between vertices have been defined by Berry et al (1986) who have suggested that the relative frequencies of primary (Va) and secondary (Vb) vertices determines the branching pattern of a given tree. They have found from their computer modelling studies of the growth of Purkinje cell

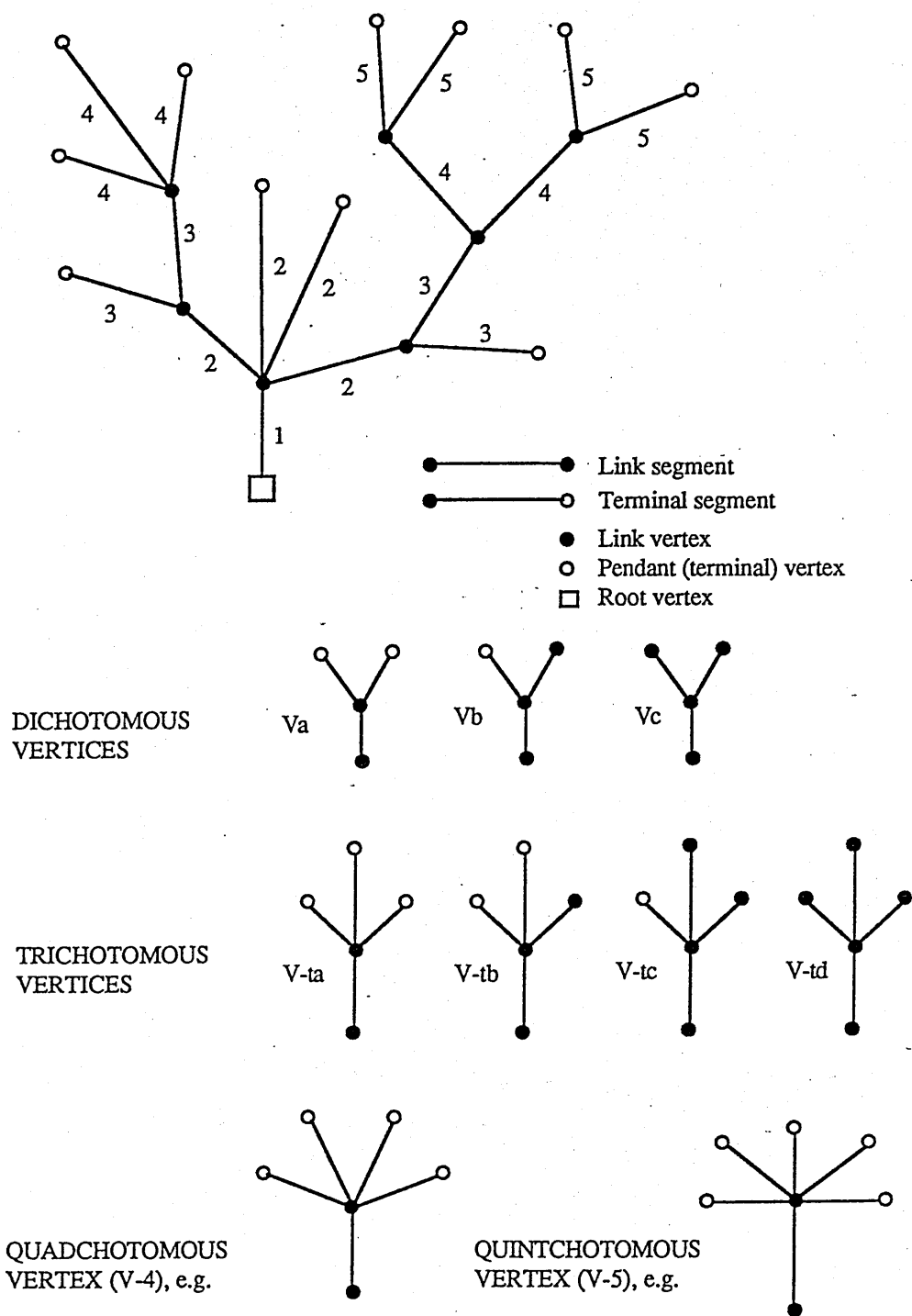


FIG. 23 . The various vertex types found and analysed in the dendritic trees of LMPNs from trained and control chicks and the centrifugal ordering system used. Dichotomous vertices Va, Vb, and Vc give rise to 2+0, 1+1, and 0+2, terminal + link segments, respectively. The number of terminal segments in trichotomous vertices V-ta, V-tb, V-tc and V-td are 3, 2, 1 and 0, respectively, and the number of link segments are 0, 1, 2 and 3, respectively. Examples of quadchotomous and quintchotomous topologies are also given. The schematic of a dendritic tree above has, for example, 2 Va's; 2 Vb's; 1 Vc; 1 V-ta; and 1 V-4.

dendritic trees, that large dichotomous networks will have equal frequencies of V_a and V_c , so that V_b is the only variable. Thus they have used the ratio V_a/V_b (the vertex ratio, V_r) to classify the modes of growth of Purkinje cell trees in terms of random terminal growth (which would have a V_r approximating 1.0), and random segmental growth (which would have a V_r approximating 0.5). They also suggest that trichotomous vertices can be incorporated into this analysis, by transforming all trichotomous vertices into combinations of dichotomous vertices, to obtain an overall V_r . This ratio, however, cannot be used in this study, because of the relatively small size of the dendritic trees of LMPNs which do not have large numbers of V_a and V_b vertices to provide a meaningful vertex ratio. Also, some of dendritic trees of LMPNs have complex vertices which give rise to 4 and 5 dendritic segments ("quadchotomous vertices" and "quintchotomous vertices", respectively) (FIG. 23). These vertex types cannot be easily adopted into the vertex ratio, and Berry et al (1986) had not considered these topological forms in their computations. An example of a neuron with a quadchotomous vertex is shown in FIG. 11 (arrow).

Therefore only the frequencies of the various topological types present on LMPNs from trained and control chicks were counted and analysed here. The total frequencies of occurrence of each of the different vertex types on all of the dendritic trees from 8 trained and 8 control chicks were collated (Table 11, appendix A). The frequency per neuron was calculated and is plotted for the different vertex types in FIG. 26 (see also Table 11, in appendix A). Statistical analyses were performed with a chi-square on the total frequencies and the dichotomous and trichotomous vertices were analysed separately.

The identification of trichotomous vertices were made on the basis that if the distance between the points of origin of the three branches was less than $1\mu\text{m}$, then they were considered to be from the same vertex node, and therefore classified as trichotomous. Similarly for the identification of quad- and quint- chotomous vertices. There are five possible topological types of quadchotomous vertices and six types of quintchotomous vertices. Because the frequencies of these types were low (see table 11), no attempt was made to separately quantify these types.

5.3 RESULTS.

NUMBER OF BRANCHES. The data from the cells in left and right hemispheres of trained and control chicks are shown in FIG. 24 and Table 8 (appendix A). Training had no significant effect on the number of branches of each order in either the left or right hemispheres (Table 8, appendix A, shows no significant effects of training or hemisphere). As expected, there was only a significant order effect, which is evident from FIG. 24. However, there are consistently more branches at each order of dendrite in the left hemisphere after training (FIG. 24b), and comparatively little change in the right hemisphere (FIG. 24a).

SHOLL ANALYSIS. Table 9 (appendix A) shows the results from the ANOVA analysis. No significant effects of training or hemisphere, or the interaction of these factors, were found. As is evident from FIG. 25, there is a significant ring effect (Table 9), but no training by ring effect. However, FIG. 25 a and b, shows that there are consistently more ring interactions per tree after training, particularly in the left hemisphere, such that an asymmetry (nonsignificant) is present in the trained chicks (FIG. 25d), but is absent in the controls (FIG. 25c).

TOPOLOGICAL ANALYSIS. No significant differences in the frequencies of the

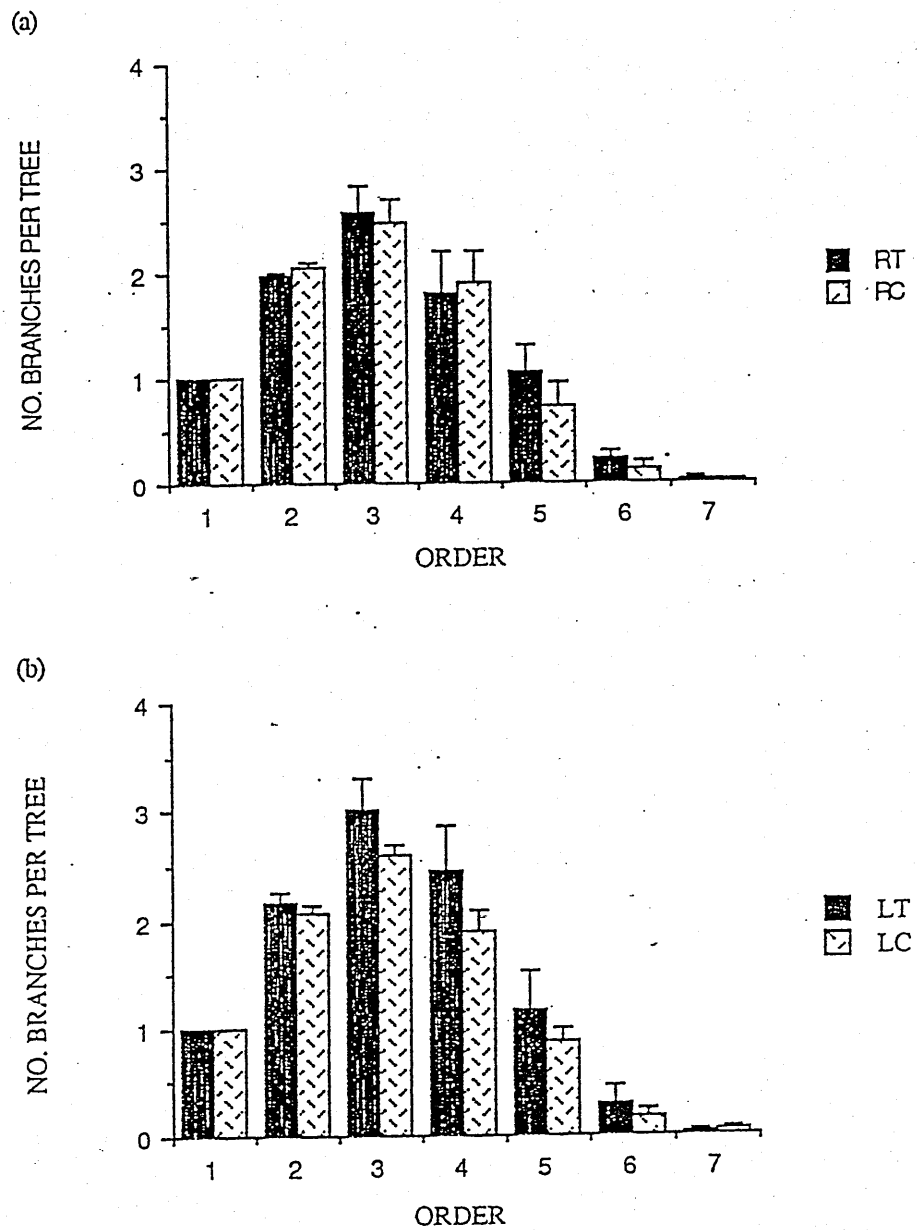


FIG. 24. Changes in the mean number of branches per dendritic tree for each order of dendrite from the cell body as a result of training. (a) Right hemisphere, (b) Left hemisphere. No significant effects of training are present.

ANOVA results are given in TABLE 8, appendix A.

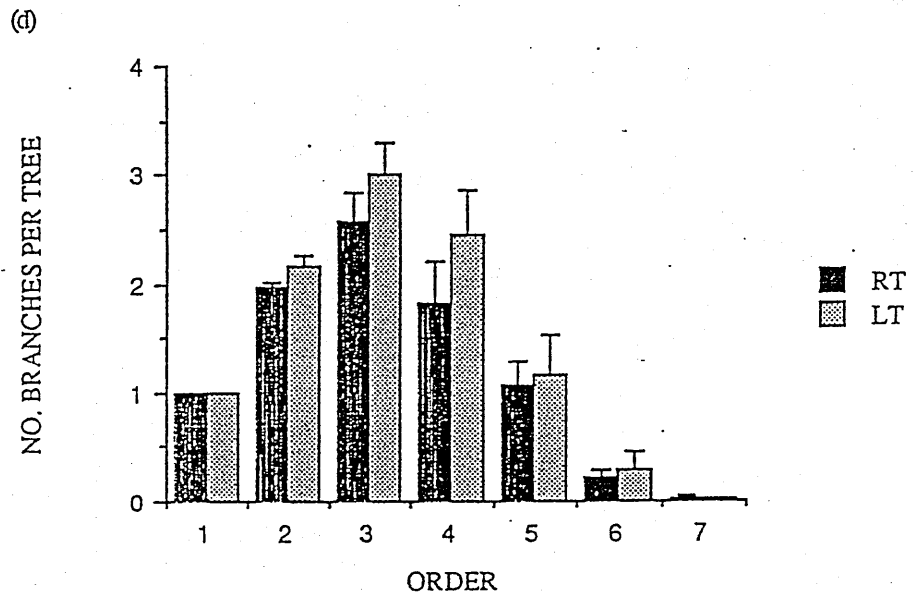
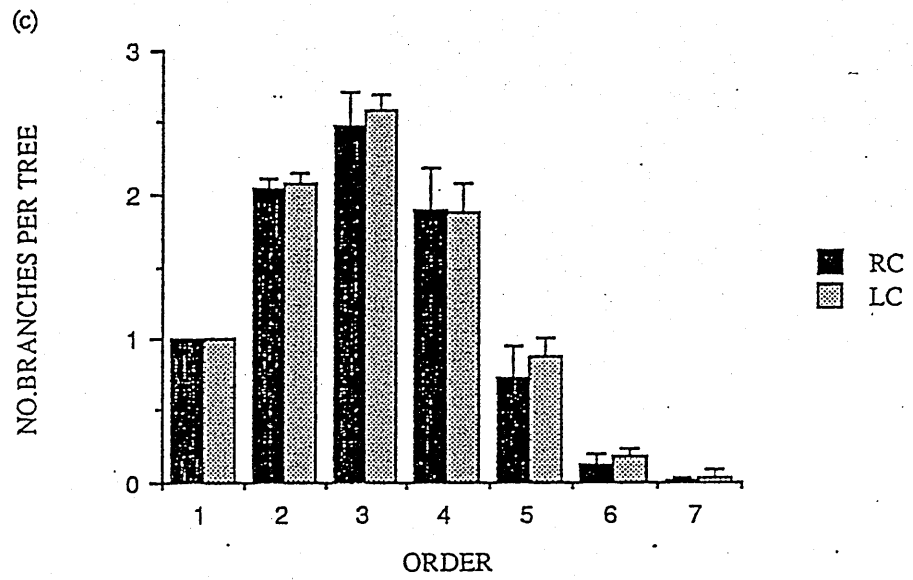


FIG. 24. Changes in the mean number of branches per dendritic tree for each order of dendrite from the cell body as a result of training: hemispheric asymmetries. There were no significant hemispheric asymmetries in control (c) and trained (d) birds. (SEMs are from 8 trained and 8 control chicks).

ANOVA results are given in TABLE 8, appendix A.

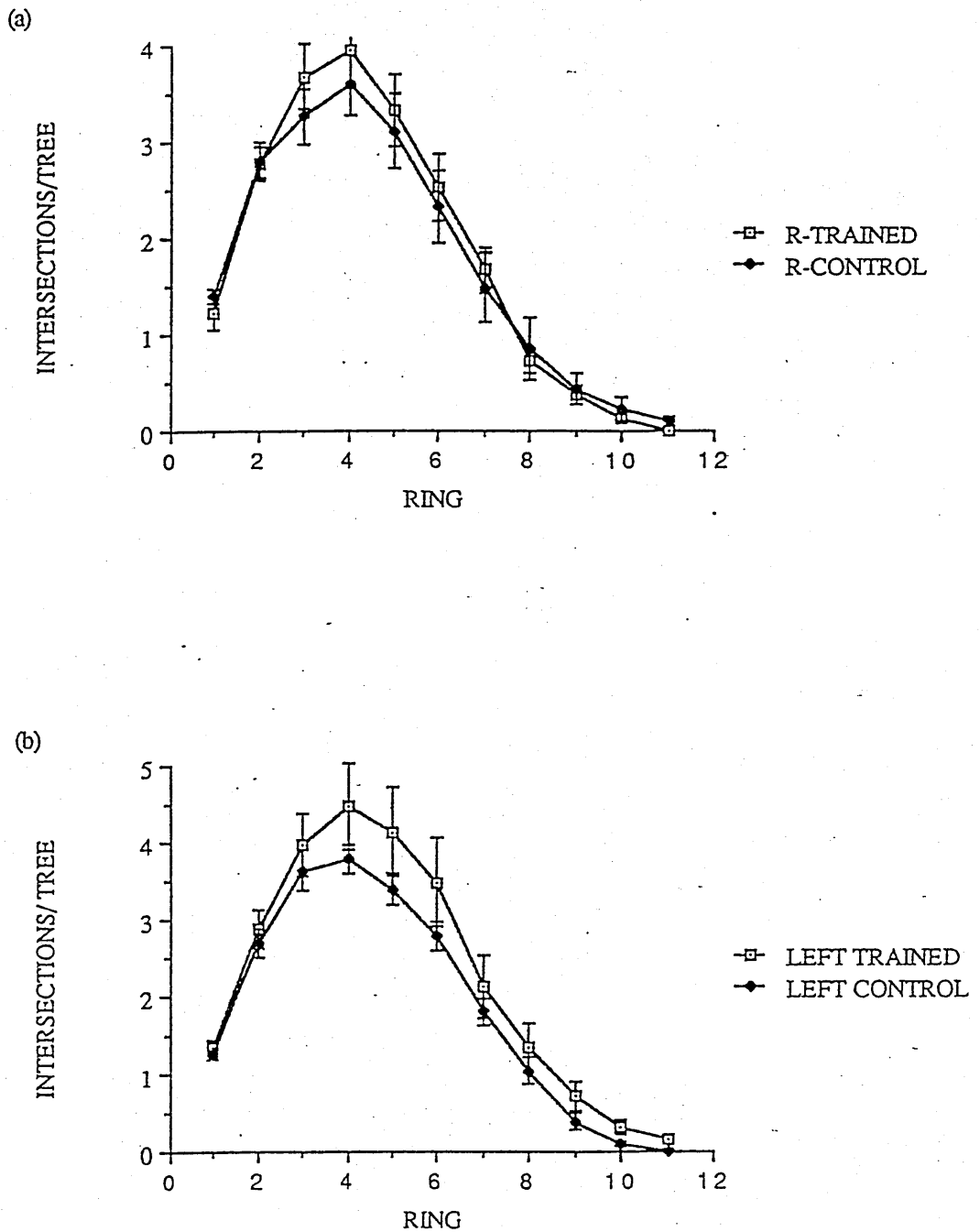


FIG. 25. Graph showing the effects of training on the mean number of ring intersections per dendritic tree plotted against the ring level numbered from the cell body. (a) Right hemisphere, (b) left hemisphere. There were no significant effects of training or hemisphere (by ANOVA), but there was a significant ring effect which is evident from the figure. (SEM's are from 8 trained and 8 control chicks). ANOVA results are given in TABLE 9, appendix A.

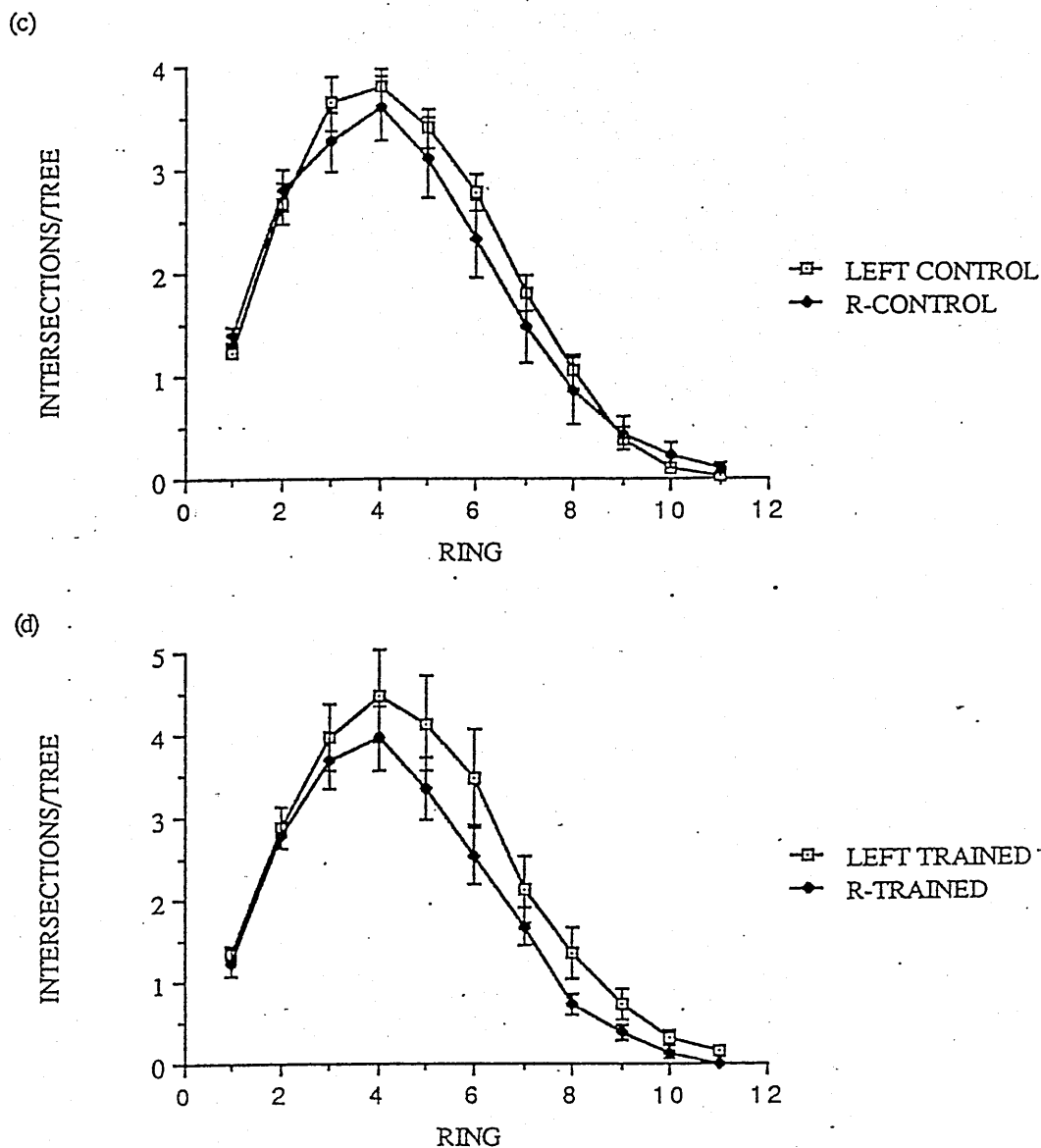


FIG. 25. Graph showing the effects of training on the mean number of ring intersections per dendritic tree plotted against the ring level numbered from the cell body: hemispheric differences. There were also no hemispheric asymmetries in control (c) and trained (d) birds. (SEM's are from 8 trained and 8 control chicks).

ANOVA results are given in TABLE 9, appendix A.

various topological types were found between trained and control chicks (Table 11). FIG. 26 gives a plot of the total frequencies per neuron of the vertices from left and right, trained and control chicks. The analysis shows that the numbers of *dichotomous* vertices on dendritic trees from LMPNs in the left hemisphere of trained chicks are greater ($V_a = 17.2\%$; $V_b = 22\%$ and $V_c = 27\%$) compared to left hemisphere controls (FIG. 26a). However, the frequencies of *trichotomous* vertices all tend to be higher in the left control than the left trained chicks by between 16% (in V_{-ta}) and 145% (in V_{-td}) (FIG. 26b).

One important observation is that the numbers of *complex* vertices (quad- and quint- chotomous) of the left hemisphere are much higher in trained compared to control chicks (FIG. 26c) and almost all of which tend to give rise to *2nd order* dendrites. Perhaps this is indicative of dendritic growth at the link vertices or segmental growth of new dendrites which are very close to the link vertices.

In contrast to these large differences in the left hemisphere, there is no apparent change in the numbers of *dichotomous* vertices between trained and control chicks in the right hemisphere (FIG. 26c). Although the neurons in the right hemisphere of trained chicks have more *trichotomous* vertices than those from the controls, these differences between trained and control chicks are much smaller in the right hemisphere than they are in the left, as shown in FIG. 26b. Further, the direction of change in the frequencies of trichotomous vertices is opposite for the left and right hemispheres: the left hemisphere of controls has a higher number of these types of vertices than the left hemisphere of trained chicks. In the right hemisphere, the trained chicks have a higher frequency of trichotomous vertices than the control chicks and the frequencies of *complex* vertices are lower in the right hemisphere

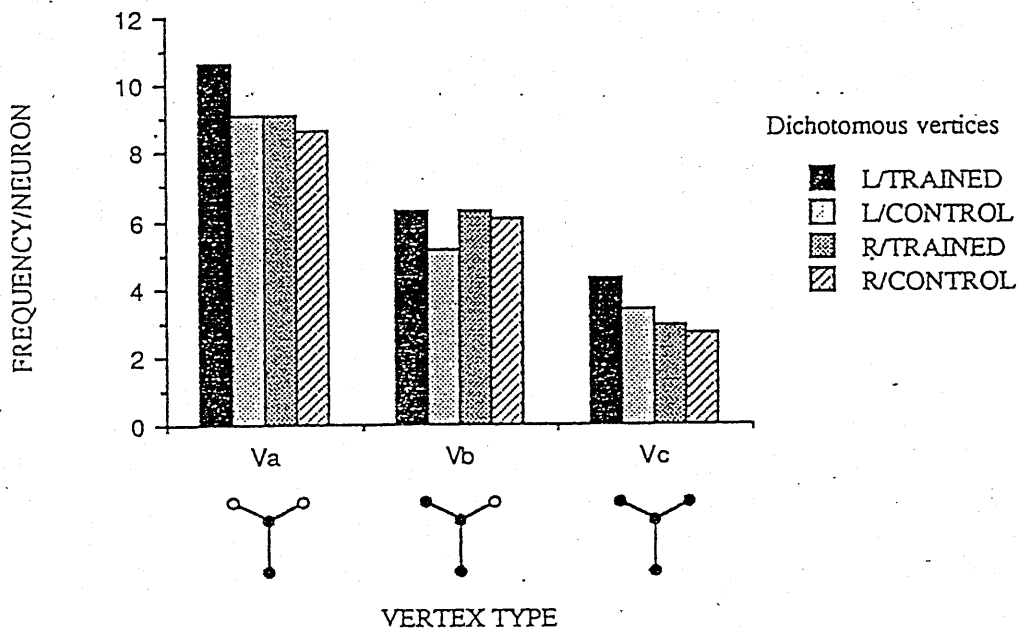


FIG. 26a. A topological analysis of the total numbers of different types of dichotomous vertices found on LMPNs from the left and right hemispheres of trained (number of neurons=52) and control (number of neurons=51) chicks. The left hemisphere shows the largest differences after training: more dichotomous vertices are found on LMPNs in the left hemisphere after training compared to any of the other groups. Therefore the frequency of segmental and terminal growth seems to have increased as a result of training. However, these differences were not found to be significant by chi-square. The data are tabulated in Table 11, in appendix A, which also gives the total frequency values.

L=left hemisphere, R=right hemisphere.

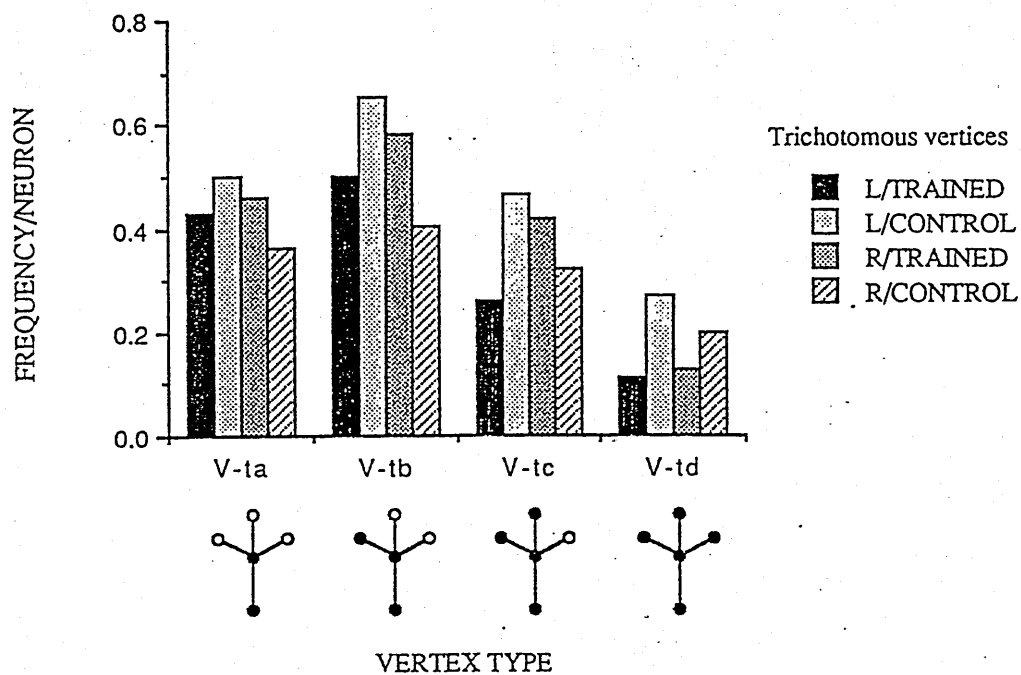


FIG. 26b. Frequencies per neuron of the different topological forms of trichotomous vertices (examples of which are given in the schematic diagrams) from 52 trained and 51 control chicks. No significant differences were found between the trained and control chicks, although the left hemisphere of control chicks tends to have the highest proportion of all of the types of trichotomous vertices. The data are also given in table 11, appendix A. R=right hemisphere, L=left hemisphere.

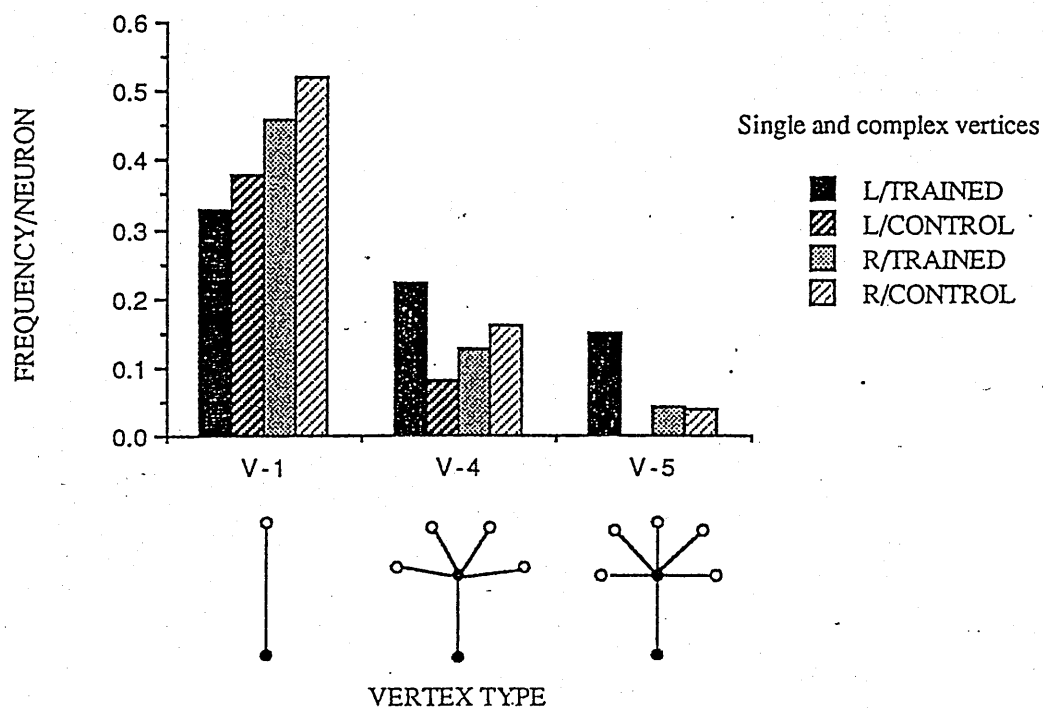


FIG. 26c. The total frequencies per neuron of single (V - 1) and complex [quadchotomous, V - 4 and quintchotomous, V-5] vertices on the dendritic trees of 52 LMPNs from trained and 51 from control chicks. The data from the hemispheres are given separately. No significant differences between the trained and control groups were found. Table 11 in appendix A gives the actual values and the frequency data. R=right hemisphere, L=left hemisphere.

than the left. In general the training differences in the right hemisphere are much smaller (FIG. 26), and are also not significant.

In addition, the frequency of dichotomous vertices is higher in the left-trained hemisphere, particularly in the numbers of Va's and Vc's (FIG. 26a), than in any of the other three groups. There are more trichotomous vertices in the left-control group than in any of the other groups, and notably, the hemispheric asymmetry in controls is marked with this variable (see FIG. 26b). The numbers of complex vertices are also higher in the left-trained group compared to the others, and as mentioned above the numbers of single vertices are much higher in the right hemisphere of control chicks (FIG. 26c).

The frequencies of *single* vertices (i.e. root vertices which do not produce any branches and simply stretch out into the tissue) also does not change significantly after training, although it is interesting to note that the numbers of these types of vertices are lowest in the left hemisphere of trained chicks, and highest in the right hemisphere of control chicks (fig. 26c). The right-control hemisphere has a lower frequency of dichotomous and trichotomous vertices, compared to the other groups, indicating that the LMPNs of this hemisphere have a less complex dendritic arborisation.

5.4. DISCUSSION / EXPERIMENT 3 .

The data presented here may be compared to earlier investigations in mammals which also examined dendritic branching changes following various experimental manipulations (see discussion on pp 86-89). For example, in contrast to the present results, the earlier studies showed more complex branching of visual cortex

pyramidal cells of rats reared in enriched environments compared to those reared in isolation or in groups (Greenough and Volkmar, 1973; Greenough et al, 1973). Similar changes were observed in maze-trained rats (Greenough, Juraska and Volkmar, 1979). Recent studies using the 2D approach have found significant alterations in branching patterns of hippocampal neurons (together with spine density changes) in rats that have been trained on an operant conditioning task for up to 7 days (Mahajan and Desiraju, 1988). Thus, this 2-dimensional method can give useful results regarding any memory related changes in dendrites. The fact that no significant alterations in branching complexity were found 25h after passive avoidance training may be interpreted in one of three ways: (1) that the branching pattern of dendrites on the LMPNs are not important for memory storage for the passive avoidance paradigm; (2) that there may be changes, but these have not been detected because of the 2-dimensional methods used or because they are too small to be noticed, and (3) that alterations in dendritic branching patterns may take longer than 25h.

Sectioning the tissue may also have affected the data, in that parts of the dendrites could have been truncated. However, these truncation effects have been partly controlled for by the fact that the tissue from both the trained and control chicks was treated similarly and sectioned into 120 μ m thick sections, so that truncation will have affected the tissue from both of the groups to a similar extent. It is unlikely that truncation will differentially affect the tissue from trained chicks more so than that from controls, or vice versa.

Although there were no significant quantitative changes in the numbers of branches of each order of dendrite per dendritic tree or in the numbers of ring intersections per tree some qualitative comparisons are still possible. There is an overall increase

of about 19.6% in the number of branches per tree in the left hemisphere after training, and this is clearly reflected in the larger number of ring intersections per tree as a result of training in the left hemisphere, shown in FIG. 25b. The dendrites tend to extend further away from the cell body of left IMHV neurons after training, as shown by the greater number of ring intersections at the 8th to the 11th levels (FIG. 25b). The numbers of dichotomous and complex vertex types have increased in the left hemisphere after training, compared to controls, with a correspondingly lower number of single vertices. Almost all of the complex vertices give rise to 2nd order dendrites, suggesting that branching is more frequent in the proximal parts of the dendritic trees of cells in the left hemisphere after training. The higher numbers of dichotomous vertices and the correspondingly lower frequency of trichotomous vertices may imply a conversion of trichotomous vertices into the dichotomous and complex vertex forms (see FIG. 27). The greater frequency of Va vertex types also suggests terminal growth of dendrites in the left hemisphere after training. Thus, the combined effect is of an apparent overall *expansion* of the dendritic field size and pattern of the neurons in the left IMHV as a result of training.

In contrast to this expansion of the dendritic field size of left IMHV neurons following training, there is no apparent significant qualitative difference in the field size of neurons in the right IMHV after training. The overall number of branches per tree seems to have increased by only 5.4%, and there is a small change in branching complexity as judged by the ring intersection analysis (FIG. 25a), as a result of training. The absence of a difference in the numbers of dichotomous vertices and complex vertices in the right hemisphere of trained chicks, compared to controls, suggests that no major change in the branching patterns have occurred in this hemisphere, although the frequency of trichotomous vertices is higher in the

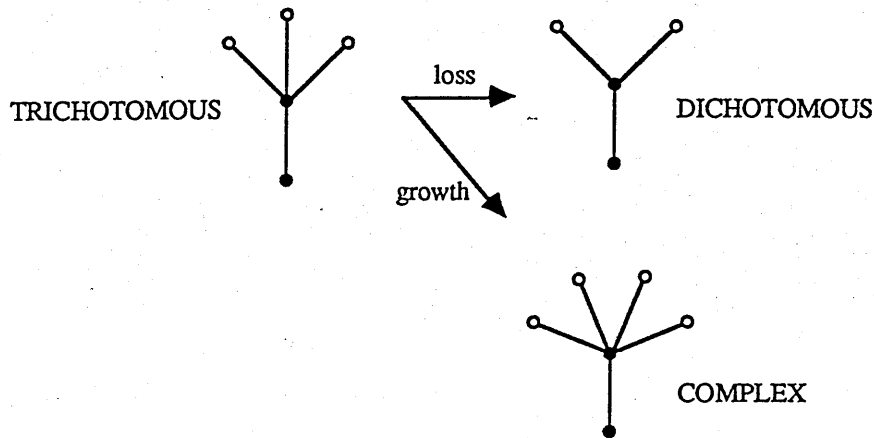


FIG. 27. Two processes of remodelling: loss of dendritic branching which could be due to the resorption of dendrites into the vertex, and growth of new branches from the vertex node possibly occurring at a different site on the dendritic tree. Both of these processes could be ongoing in the left hemisphere after training, because there are less trichotomous vertices, in this hemisphere in trained chicks, with a corresponding higher frequency of dichotomous and complex vertices (FIG. 26).

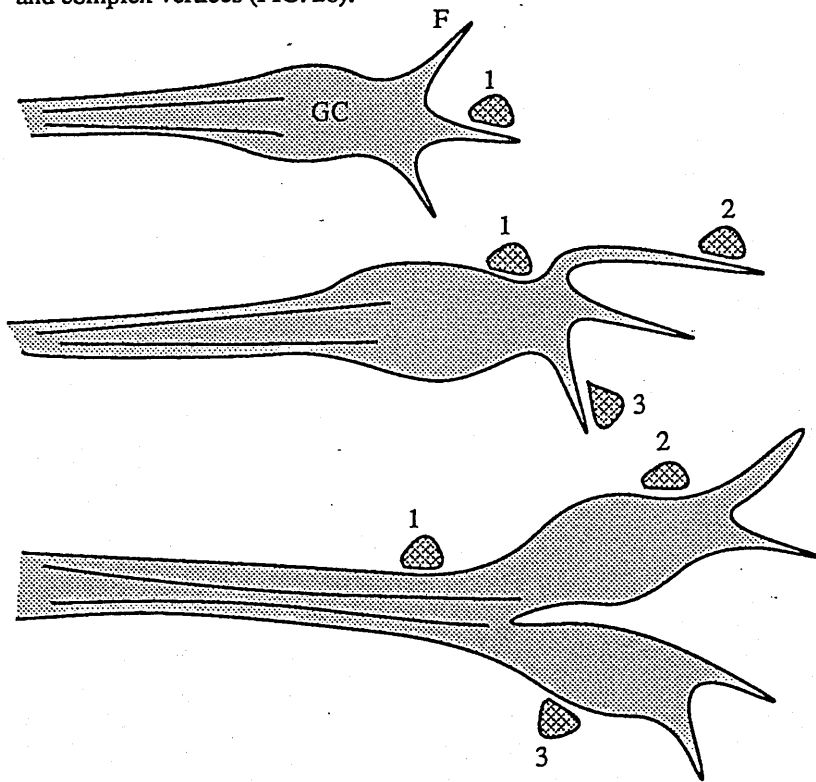


FIG. 28. Filopodial synaptogenetic hypothesis of dendritic growth (Vaughn et al, 1974). D, dendritic shaft; GC, growth cone; F, filopodium. A single filopodium attaches to axon terminal 1, which becomes translocated onto the growth cone and the dendrite shaft as the growth cone advances. The growth cone divides where filopodia have contacted axons 2 and 3, thereby establishing a branch point.

right-trained hemisphere. However, the fewer ring intersections at the higher levels (8th to the 11th), and the correspondingly higher number at the lower levels (FIG. 25a), suggests a net contraction of the dendritic field size of LMPNs in the right IMHV. Interestingly, the change in frequencies of the different types of vertices in the right control hemisphere is in an opposite direction to the changes in the left hemisphere after training. Trichotomy is greater in the left-control and right-trained hemispheres and lower in left trained and right control. The numbers of dichotomous, trichotomous and complex vertices are, in general higher in the left-trained hemisphere, whilst the numbers of single vertices are higher in the right hemisphere of control chicks.

Thus, the dendritic branching patterns of LMPNs in the left hemisphere of trained chicks appear to be much more complex than those of the left control hemisphere, and indeed those from the other groups also. The right hemisphere shows relatively little change in any of the branching parameters, and the directions of the differences suggest a less complex arborisation pattern after training compared to right hemisphere controls, and the left hemisphere, after training.

These qualitative changes need to be tested directly, however, with a proper three dimensional analysis of the dendritic trees, before any firm conclusions can be drawn regarding the dendritic field size changes following training. Even so, the apparent hemispheric asymmetry is of importance in that it is highly consistent with other parameters that have been measured to date, and therefore cannot be ignored. This will be discussed below at greater length (in the final discussion).

Gross changes in the patterning of the dendritic trees of neurons that also show

large increases in the spine density as a result of passive avoidance training suggests more dendritic material has been manufactured in these neurons (LMPNs). It would be reasonable to infer, therefore, that new synapses are possibly forming. The larger numbers of complex vertices in the left hemisphere of trained chicks may indicate that growth of new branches has occurred at trichotomous vertices (FIG. 27).

The mechanisms underlying this growth pattern, however, can only be speculated upon. It is evident that growth of new dendritic branches occurs, whilst others are retracted (see introduction). This implies that growth cones remain functional throughout life. There is some evidence in the literature suggesting that growth cones remain functional in older animals. Starving rats for 30 days has the effect of contracting the field size of Purkinje cell dendritic trees. Upon refeeding the rats from 30 to 80 days, the lengths of the dendritic segments increase and the topological pattern of the Purkinje cell dendritic trees also changes. Both terminal and nonterminal segments underwent elongation suggesting that terminal and segmental growth cones are functional in these animals (McConnell and Berry, 1978a, b). The most likely mechanism by which the dendritic trees take on a particular pattern during development, is that presented by Vaughn et al (1974) who refer to it as the "filopodial adhesive hypothesis". Berry et al (1978) have renamed it as the "filopodial attachment hypothesis" of the growth of dendritic branches on Purkinje neurons, because the contacts between axons and dendrites are not exclusively synaptic. Such a mechanism can be used to perhaps explain the growth and retraction of branches in older animals. Berry et al (1978) have also suggested that the growth of Purkinje cells occurs in two phases: the initial growth being genetically controlled, and a secondary phase which is determined by the action of the axonal fields around the growing dendrites (Berry et al, 1978; Berry et al,

1980). The hypothesis states that during development, filopodia at the growth cone site randomly extend out into the neuropile and establish synaptic contacts with axons *en passant* (FIG. 28). The synapse is then translocated onto the growth cone and dendritic shaft as the growth cone advances. Branching occurs when two or more synapses are formed with as many filopodia, such that the growth cone divides itself into two or more growth cones, as shown in FIG. 28.

The influence of afferent fibres on the growth of dendrites has been discussed by Berry et al (1980), who conclude that the establishment of a critical number of parallel fibre synaptic contacts with Purkinje cells during the early postnatal period in the rat, may be necessary for the secondary induction of growth of the Purkinje cell dendritic tree (Berry et al, 1978) (see also chapter 2). Dendritic remodelling can also occur later in development as many studies on the effects of deafferentation (e.g. Jones and Thomas, 1962; Bradley and Berry, 1976a, b; Berry and Bradley, 1976a) and subsequent regrowth have shown (e.g. Caceres and Steward, 1983; Steward and Vinsant, 1983). (The effects of stimulation and environmental enrichment on dendritic branching patterns have been discussed in the introduction to this chapter).

Thus, the axonal innervation that a given neuron receives, has a profound influence on the development of the neuron's dendritic field, and its subsequent maintenance in later life. That patterns of neuronal activity, as opposed to general non-specific activity, are necessary for the induction of morphological changes in the postsynaptic target neuron, has been partly demonstrated by Black et al (1987). They show that the number of synapses on Purkinje cells alters as a result of learning and not simply because of non-specific activity resulting from exercise.

This has important implications for understanding the mechanisms involved in learning and memory, a discussion of which is given in chapter 6. As depicted in FIG. 27, a remodelling of the dendritic trees seems to have occurred following learning, involving a loss of some branches and a new growth of other dendrites. The results of computer simulation studies, show that this remodelling may alter the physiological properties of the LMPNs (Rall and Segev, 1987). For example, the effectiveness of synapses located on distal dendrites can also be enhanced by the spread of current from one spine to another (Shepherd et al, 1985) (see "function of dendritic spines in chapter 2).

CHAPTER 6

GENERAL DISCUSSION: DENDRITIC SPINES AND MEMORY FORMATION IN THE CHICK

The series of experiments reported in chapters 3-5 show specific increases in the spine densities of large, multipolar projection neurons (LMPNs) in the chick IMHV, 25h after passive avoidance training (FIGS. 15 and 21). Although the neurons from both hemispheres show these increases, only the LMPNs of the left IMHV show significantly enhanced spine densities which are specifically related to the memory for the task as demonstrated by the electroshock experiments. Changes in the shape of dendritic spines, such as spine head diameter enlargement (FIG. 19) and spine stem length shortening (FIG. 20) have been observed only in particular dendrite branch orders and these are also lateralised to the left IMHV. The alterations occur without any significant change in the branching patterns of the LMPNs after training, as analysed by the ring intersection method (FIG. 25), the number of branches of each order from the cell body (FIG. 24) and the frequencies of the various topological branching patterns (FIG. 26). No significant changes in the mean lengths of the different dendrite branch orders (FIG. 17), their dendrite diameters (FIG. 16) and in the mean overall spine lengths (FIG. 18) were obtained. Qualitatively, the major changes in branching patterns occurred mainly in the left hemisphere: the topological analysis showed that the branching patterns of LMPNs were more complex in the left hemisphere after training (FIG. 26), with little change in the right hemisphere. There were also more branches of each dendrite order (FIG. 24), and correspondingly higher number of ring intersections with dendritic branches of the LMPNs from the left hemisphere following training (FIG. 25). The effects of training were also less marked in the right hemisphere. Because

training seems to have affected the left hemisphere to a greater degree than the right, some hemispheric asymmetries in trained chicks are apparent: compared to the right hemisphere, the left hemisphere has a greater number of branches of each order per tree (FIG. 24); more ring intersections per tree (FIG. 25); a higher frequency of complex and dichotomous vertices (FIG. 26); thinner dendrites of orders 2-4 (FIG. 16); longer spine stems (FIG. 20); and from the electroshock experiment, a significant increase in spine density that is specifically related to memory formation (FIG. 21).

A significant hemispheric asymmetry in the spine density of control chicks was also found, with the LMPNs of the right control hemisphere having a greater spine density than the left. Training had significantly increased the spine densities in both hemispheres such that no hemispheric asymmetry was present in the trained birds. The fact that the spine densities on all of the dendrite branch orders in both hemispheres have reached the same level as a result of training, suggests that it is a property of the entire neuron, and that this post-training level may well be the maximum. It does not, however, appear to be due to a general trophic effect on the neuron because no significant changes in most of the other dendritic parameters were detected (i.e. number of ring intersections; dendrite lengths and dendrite diameters; and the topological parameters). Perhaps large differences in branching patterns are not observable 24h after training - a longer time course study will probably determine this.

One possible interpretation of the present results, would be to attribute the changes in spine number and shape to developmental mechanisms, whereby in the one day old chick, the LMPNs in the right IMHV are morphologically more mature than those in the left, as judged by the fact that the spine density in the right control

hemisphere was found to be greater than that in the left (experiment 1: FIG. 15c). The rate of development of the left hemisphere may be enhanced by the increased neuronal activity occurring as a direct consequence of the training experience, which then "catches up" with the right hemisphere. Indeed, from the arguments presented in the introduction (chapter 2), the factors governing the growth of dendritic spines are both genetic and environmental. Therefore there is no *a priori* reason to assume that both of the hemispheres will develop at the same rate and to the same extent. However, if this were the case, then the apparent immaturity of the left IMHV should be revealed by other morphological parameters. From the dendritic branching data, there were no significant changes in either hemisphere after training and there were no hemispheric asymmetries in these parameters in the controls. If anything, the LMPNs of the left hemisphere tended to have more higher order branches per dendritic tree compared to the right, after training (FIG. 24d). This is also reflected in the number of ring intersections per dendritic tree (FIG. 25d). Further, the 2nd and 3rd order dendrite branches are significantly longer in the left control hemisphere than the right (FIG. 17), and almost all of the dendrite segments of the left control hemisphere tend to be thicker than those in the right (FIG. 16). In addition, there were no significant changes in overall spine length in the control hemispheres or after training (FIG. 18). Thus there is no direct support for the suggestion that the LMPNs in the right IMHV are developmentally superior to those in the left, in the one day old chick.

6.1. SPECIFIC INCREASES WITHIN 25H.

One of the main findings from these studies is that increases in spine density can occur within a 24h time period and *specifically* in response to a one-trial learning event. This is in line with the amnesia experiments which show that the increases in

spine density are specifically related to memory storage processes. Previous investigations of this subject have only revealed alterations in the number of spines and dendrites after compound training experiences that have been stretched out for days to months (discussed in chapters 2 and 5). For example daily operant conditioning for 7 days (Maharajan and Desiraju, 1988); maze-training for up to 30 days sometimes with enriched rearing conditions (Greenough et al, 1979; for review see Greenough and Chang, 1985). In further contrast to these reports, no changes in dendritic branching patterns were found after passive avoidance training, suggesting that spine density alterations can indeed occur without an overall gross alteration of the entire neuron, which is suggestive of a specific and co-ordinated development of the neuron as a result of the training experience.

It is possible for the morphology of CNS neurons to be altered within 24h because the rate of dendritic transport of 100mm/day (Kiss, 1977) will allow newly synthesised proteins from the large dendrites and the perikarya to be passed to distal dendrites (which may be about 150µm from the soma) within 2-2.5 min. Also, polyribosomal aggregates have been found in the head, stem and base areas of spines in electron microscopic investigations of the visual cortex, indicating that proteins may be formed at the sites at which they may be used (Greenough, 1986). Greenough (1986) and Steward (1983) argue for the presence of these aggregates within dendritic spines, as indicators of newly formed synapses, because the percentage of spines with polyribosomal aggregates increases in those animals reared in enriched conditions compared to those in isolated conditions.

6.2. SPINE INDUCTION BY NEURONAL ACTIVITY.

From the arguments presented in chapter 2, it is evident that the formation of dendritic spines during early development may have two phases: an initial stage that

is genetically controlled (or induced during the embryonic period), and a second stage that is environmentally determined. Activity of the neurons as a result of sensory stimulation and learning can induce the growth of a secondary phase of dendritic spines during development (see chapter 2). For the passive avoidance learning task, enhanced "bursting" activity has been recorded in anaesthetised chicks up to 12h after training in both left and right IMHV regions (Mason and Rose, 1987). This activity is abolished in chicks that had been given the electroshock treatment described above, immediately after training, and which were rendered amnesic as a result (Mason and Rose, 1988). Thus, from these results it can be postulated that the greater activity of the neurons in the IMHV after training could have induced or initiated the secondary phase of formation of dendritic spines. Depolarisation induced by stimulation from other afferent fibres which may be coactive at the time or indeed other extracellular agents, such as the catecholeamines and neuromodulatory hormones, may be required in order for morphological effects to be induced in a particular neuron. High frequency activity (similar to "bursting" activity) is known to induce spine growth as shown by *in vitro* studies of LTP in hippocampal slices. For example, Chang and Greenough (1984) show that high frequency stimulation of the perforant path which projects to dentate granule cells (FIG. 3) results in an increase in the numbers of shaft and sessile spine synapses in this region (see also pages 30-33).

Mattson (1988) has recently reviewed evidence suggesting that neurotransmitters released by afferents innervating particular cell populations can have morphological effects on the structural development of the target neurons. He has proposed that the release of neurotransmitters, and the activity of the neurons in later life, could also have marked effects on the innervated neurons, such as changing the structure

of the activated synapses, altering the branching patterns of dendrites on specific cells and inducing the growth of dendritic spines. If this were the case, then we would have a plausible mechanism by which "bursting" activity, occurring as a result of learning, could induce structural alterations on neurons that may be involved in the storage of the engram.

Studies of cell culture have shown that neurotransmitters can affect the morphology of different neurons in quite specific ways. For example, Baloyannis et al (1983) cultured rat cortical pyramidal cells and cerebellar neurons excised from newborn rat pups, and found some synapse-free dendritic spines on both cortical pyramidal cells and cerebellar Purkinje neurons. They also observed that the density of spines can be increased if GABA or glycine was enriched in the culture medium, and decreased if sodium barbiturate was present. Recent studies reviewed by Mattson (1988) demonstrate that neurotransmitters and electrical activity can regulate, in quite specific ways, neuronal outgrowth and synaptogenesis. For example, Cohen and Kater (1986) induced action potentials in isolated *Helisoma* neurons in culture, which resulted in a retraction of the filopodia and lamellipodia, and a cessation of neurite elongation; outgrowth resumed following removal of the stimulus. Similarly, exposing cultured pyramidal neurons to a medium containing elevated levels of K^+ (a condition causing membrane depolarization), the outgrowth rates of both the axon and dendrites were reduced (Mattson et al, 1988). Several other *in vitro* studies have also shown the effect of electrical activity on the cytoarchitecture of the postsynaptic cell, and the outgrowth of neurites (Hinkle et al, 1981; Jaffe and Poo, 1979; Marsh and Beams, 1949; Patel and Poo, 1982).

Whether neurotransmitters can affect the cytoarchitecture of specific cells depends upon the presence of receptors on the cell surface. It is interesting to note that the

arrival of afferents to a particular cell population during development coincides with the sensitivity of those neurons to specific neurotransmitters. This is well demonstrated in the cerebellar Purkinje neurons whose continued growth during development is dependent upon the arrival of parallel fibre afferents (discussed in chapter 2); and in the hippocampus. Synapses in cultured hippocampal neurons only form in an axodendritic arrangement (Bartlett and Banker, 1984). These cultured neurons extend one long axon and several short dendrites, which are morphologically and structurally distinct. Mattson et al (1987, 1988) have shown that glutamate, focally applied in a dose dependent manner to the cultured cells, causes only a reduction in dendritic length, but has no effect on axonal growth, presumably because the axons lack functional glutamate receptors. Maximum sensitivity of these neurons to glutamate was seen by day 4 in culture and only the kainate/quisqualate receptor types are involved at this stage (NMDA sensitivity develops over a 1-2 week period in culture). Addition of the inhibitory neurotransmitter, GABA and its potentiator diazepam, at days 2-4 in culture, suppressed the outgrowth of both axons and dendrites, suggesting that an optimal level of electrical activity is necessary for neurite outgrowth: excess excitation (glutamate) or inhibition (GABA-diazepam) by neurotransmitters can suppress outgrowth. Mattson (1988) also presents further evidence showing that local release of neurotransmitters from axons can greatly modify the dendritic architecture of pyramidal neurons, by placing these neurons in a mat of cultured entorhinal axons, which normally release glutamate. The dendritic arborisations of the pyramidal cells was found to have been severely diminished. Acetylcholine and norepinephrine are also capable of suppressing dendritic outgrowth in hippocampal cells (Mattson, 1988), although the effects are only seen in neurons which have been in culture for at least one week.

This development of the sensitivities of the pyramidal neurons to the different neurotransmitters at later stages of maturation can be considered in relation to the normal arrival of afferent inputs to the hippocampus *in vivo*. Entorhinal and intrinsic glutamatergic and GABAergic inputs develop first. Within the first week of birth, the septal cholinergic and the noradrenergic inputs from the locus coeruleus impinge upon the pyramidal neurons (Hyman et al, 1986; Isaacson and Pribram, 1975). These studies suggest that the outgrowth of pyramidal neuron dendrites becomes sensitive to the different neurotransmitters in a temporal sequence consistent with the times of arrival of the axons which release the neurotransmitters, *in vivo*. Therefore, the neurotransmitters released by incoming afferents may play an important role in the establishment of the laminated arrangement of synapses on the pyramidal neuron dendrites in the hippocampus (Mattson, 1988) (FIG. 3). The influence of these afferent fibres on the development of spines in the hippocampus was discussed in chapter 2 (see pages 26-28).

One may therefore ask, what are the mechanisms by which afferent fibres exert such influences on the growth of the neurons? Mattson (1988) has suggested the involvement of second messenger systems, which may be activated by the binding of neurotransmitters or other extracellular agents, to receptors on the cell surface such that they subsequently promote protein synthesis in the cell (discussed in more detail later, in relation to the mechanisms of spine shape changes). Recently, Black et al (1987) have shown that increased impulse activity can elicit a rise in RNA synthesis with a resultant elevation in tyrosine hydroxylase and norepinephrine synthesis that persists long after the exciting stimulus has been removed. Thus it is possible for an increase in protein synthesis to occur upon, for example, "bursting" activity that has been observed in the IMHV after passive avoidance training. If this

is the case, then protein synthesis changes should also be observed following passive avoidance training.

Indeed, increases in ^3H -fucose incorporation into glycoproteins have been observed up to 24h after training, which together with the enhanced "bursting" activity and the spine density increases reported here, are also specifically related to memory storage processes, because amnesic chicks do not show these changes (Rose and Harding, 1984; Mason and Rose, 1988). In addition, enhanced incorporation of ^{14}C -leucine into tubulin following training has also been reported (Mileusnic et al, 1980). Together, tubulin, which is a major component of the spine cytoskeleton (see FIG. 30) and glycoproteins, which are major constituents of neuronal membranes, may well be used in the production of dendritic spines after training.

This is substantiated by the observation that protein synthesis inhibitors such as anisomycin and emetine, are very effective in causing amnesia when administered intracranially into the forebrain (Gibbs and Ng, 1977) or locally into the IMHV (Patterson et al, 1986), within a time window of 15 min before, to 15 min after passive avoidance training. Patterson et al (1986) have also found hemispheric asymmetries with these studies: emetine, which can inhibit protein synthesis locally at the site of administration, only has amnesic effects when injected either bilaterally or unilaterally into the left IMHV, but not when unilaterally injected into the right IMHV. Similar results were found with the injection of ouabain, which is an inhibitor of Na^+/K^+ ATPase and glutamate. Further, Howard et al (1980) show that the amnesic actions of CXM, which is similar to that of anisomycin, may well have resulted from its effect of increasing the available pool of amino acids, such as glutamate. These experiments have tested the possibility that over-excitation of the

cells due to increased levels of glutamate causes amnesia. That glutamate may in fact be involved in some aspect of the storage process is partially indicated by the recent observation of an increase in NMDA receptor sites after imprinting in the chick, which also occurs only in the left IMHV (McCabe and Horn, 1988). It would, therefore, be interesting to test for the effects of drugs which block the actions of glutamate. That is, do glutamate antagonists, such as DGG (γ -D-glutamylglycine), cause amnesia if injected into the IMHV, and if so, are the morphological changes in spines still present 24h after training?

6.3. THE HEMISPHERIC ASYMMETRIES.

These differential hemispheric effects of protein synthesis inhibitors and neurotransmitters, which only cause amnesia for the passive avoidance training task when they are injected into the left hemisphere and more specifically into the IMHV, are also correlated with the spine shape changes and the dendritic remodelling reported in this thesis. The increases in the spine head diameter and shortening of the spine stem, were observed only in the left IMHV, 25h after training. Also, the spine density increases were lateralised to the left hemisphere, as shown by the amnesia experiment. It would be important to know if the spine density and shape changes are present in chicks that have been trained, but rendered amnesic by the administration of emetine into the IMHV, because protein synthesis appears to be required not only for the formation of new spines but also for changing spine shape. Fifkova et al (1982), show that the enlargement of the spines in the dentate molecular layer which occurs within 4 min of direct tetanic stimulation of the perforant path fibres is abolished if anisomycin is subcutaneously applied 15 min prior to stimulation.

Spine shape changes such as head enlargement and stem shortening, can also be

found within 9 min of an intense learning experience in jewel fish, and can persist for up to 24h (as discussed in the introduction). Therefore, the increases in spine head diameter and stem shortening found 25h after passive avoidance training in the IMHV, may well have been present as soon as ten min after the one-trial passive avoidance training task, a possibility which should be tested further.

The lateralised morphological effects reported in this thesis are also correlated with several other findings: 2-deoxyglucose metabolism is markedly increased in the thirty minutes after training only in the left IMHV (Rose and Csillag, 1985); the phosphorylation of a presynaptic membrane protein kinase C substrate, a 52KD phosphoprotein is also affected by training in the left hemisphere (Ali et al, in preparation) and presynaptic changes - increases in the volume density of presynaptic boutons and number of vesicles per synapse - 24h after passive avoidance training are also lateralised to the left IMHV. Qualitative observations of changes in the branching patterns of dendritic trees on LMPNs have also revealed more complex dendritic arbors in the left hemisphere of trained chicks.

In short, significantly more morphological and biochemical alterations are occurring in the left, rather than the right hemisphere, up to 24h after passive avoidance training, indicating that the left IMHV is predominantly involved in the processing and perhaps the storage of the learnt information. Behavioural experiments have provided substantial evidence for this conclusion: in addition to the pharmacological experiments discussed above, Gaston and Gaston (1984) have found that chicks trained on a pattern discrimination task with both eyes open show good retention of the task when tested monocularly through the right eye (left hemisphere), but insignificant retention when tested through the left eye (right hemisphere). Hence

both hemispheres are involved in the performance of the task, but only the left hemisphere seems to have access to the learned information. Andrew et al (1980) demonstrate that whereas the left hemisphere is particularly involved in visual discrimination tasks, the right is preferentially involved in fear responding. Birds were monocularly occluded and presented with a threatening stimulus which characteristically evokes fear and escape behaviour. Those that had their right eye (left hemisphere) occluded, evoked this flight response, whilst those that had their left eye (right hemisphere) occluded did not show these fear behaviours. The left hemisphere therefore seems to be particularly specialised for tasks involving visual and auditory discrimination learning (Gaston and Gaston, 1984; Howard et al, 1980; Mench and Andrew, 1986; Rogers and Anson, 1979; Rogers and Ehrlich, 1983; Nottebohm, 1977) and the right hemisphere for fear responding to novel objects (Andrew, 1983; Andrew and Brennan, 1983; Phillips and Youngren, 1986).

Various hemispheric asymmetries of behavioural functions have also been found in the brains of many animals, including chicks, songbirds, rodents, nonhuman primates and man and these are also correlated with morphological differences (for reviews see: Andrew, 1983; Bradshaw and Nettleton, 1981; Denenberg, 1981; Nottebohm, 1977; Walker, 1980).

Perhaps the functional importance of these hemispheric asymmetries in chicks can be best understood in relation to one of the major learning events occurring in the chick, that is, imprinting. Interhemispheric interactions (one inhibiting or activating the other - Denenberg, 1981) are likely to occur, given the differential processing capacities of the two hemispheres, discussed above. Lesion, and morphological studies, have revealed the importance of the left and right IMHV regions in this

process. Horn and colleagues (Cipello-Neto et al, 1982; Horn et al, 1983), have shown that although the memory of the mother figure may not be permanently stored in either IMHV regions, the processing of the information critically involves both the left and the right IMHV's. They found that firstly, either the left or the right IMHV must be present for imprinting to occur, and also that initial retention depends critically upon the presence of the left IMHV, whether or not the right IMHV is removed before or after training. Secondly, 26h after training neither IMHV regions are crucial for at least the recall of the learnt information, which implies that a separate extra-IMHV store (S') has been formed during this time period. However, lesioning the right IMHV 21h after training prevented the formation of S'. This implies that the store S' probably develops between 21 and 26h after training and its formation is dependent upon the presence of the right IMHV at least 3 to 21h after training: when the right IMHV was destroyed before 3h after training and then the left, the chicks were amnesic. Thus, the left and right hemispheres appear to be processing the visual and auditory information in a time dependent manner. Interactive processing of the information is perhaps important for imprinting, because the chick uses this information to identify the "mother figure" and initiate approach behaviours. The predominance of the left IMHV in visual and auditory processing and the right in controlling fear and approach behaviours, are therefore possibly phylogenetically determined, and advantageous hemispheric asymmetries, for the young chick.

6.4. BIOPHYSICAL INTERPRETATION OF THE DATA.

Interpretations of the changes in the density and shape of dendritic spines using computer simulation data, clearly supports the claim that greater information processing is occurring (or has occurred within the 25h following training) in the left

hemisphere, than in the right. From the literature reviewed in the introduction, it is apparent that dendritic spines and the dendritic branching patterns of the neurons are important information processing units, and any changes in these structures will undoubtedly alter the processing capacity of the neuron. An increase in spine density provides for the amplification of the excitatory postsynaptic signal onto a spine head, which is a very useful property if the input is located on a distal dendrite branch, and it also provides for a more efficient propagation of the impulse to the cell body (see pages 36-39) (Jack et al, 1975; Koch and Poggio, 1983; Wilson, 1984; Miller et al, 1985). Further, the amplitude of an excitatory synaptic input onto the spine head can be regulated by varying the cytoplasmic resistance of the spine stem and the spine head. Thus, for an optimal range of spine head diameter and spine stem values, the cytoplasmic resistance of the spine will be of sufficient magnitude for the amplification of the excitatory signal. Other values of these parameters will either attenuate or completely check the input. From simulations of this kind, we can theoretically interpret the 9% increase in spine head diameter and the 17% decrease in spine stem length to imply a change in the efficacy of the synaptic input, such that the signal may be amplified. However, further computer simulations of the LMPNs using the data presented in this thesis are needed to confirm these interpretations.

Synaptic efficacy changes are purported to occur upon storage of learnt information (Hebb, 1949). It has been suggested that persistent neural activity, perhaps in the form of patterned reverberatory activity within a given neural network, can lead to a change in the structure of the activated synapses thereby storing the learnt information. The information can be retrieved at a later date by re-activating these neural pathways. By converting a synapse on a dendrite shaft into a spine synapse, the modulatory power of that synapse can be increased considerably. This specific

spine induction hypothesis has been discussed previously (FIG. 4). If the synapse is already located on a spine head, its efficacy can be altered by changing the dimensions of the spine stem and head, as discussed above. Viewed in this way, we can place a better interpretation on the changes in spine density and shape observed on various neuron types (pyramidal, stellate, multipolar), in different species of birds and animals (rats, mice, monkeys, ferrets, cats, mynah birds, honeybees, jewel fish, etc) and after many different forms of learning or stimulating experiences (electrical stimulation; rearing in enriched conditions; place learning in honeybees; operant conditioning; extensive social experience or isolation in jewel fish, etc), which were discussed earlier in this thesis. Similarly for the increases in spine density and changes in spine shape observed after passive avoidance training in the chick.

6.5. NEW SYNAPSES?

Theoretically, therefore, in relation to memory storage for the passive avoidance task, the increases in dendritic spine density and the changes in spine shape may be interpreted as having a facilitatory effect on already existing synapses and/or the formation of new connections which may be important for the storage of the engram. Apart from the theoretical physiological effects of the spine density increase discussed above, the specific relation of dendritic spines to memory formation may simply be explained by the "connectionist" view (e.g. Swindale, 1981; see page 39). That is, spines may form in order to make synaptic connections with axons distant to the dendritic shaft (FIG. 4). Such a possibility implies that some dendritic spines do not have a synaptic connection prior to forming one with the distant axon. Thus, at various times after training, synapse-free dendritic spines should be observed, which are in the process of forming a connection in this way.

This would also imply that more synaptic contacts are forming after training, so that by 24-26h all or most of the dendritic spines will have a synaptic contact. This possibility is amenable to testing, by performing a time course study at the electron microscope level, but was considered beyond the scope of the present study because of the time likely to be required.

In addition, by drawing parallels with the mammalian cerebral cortex where each spine has at least one synaptic contact, this large memory-specific increase in spine density, may also represent a corresponding increase in synapse density on LMPNs. Rogers and Sink (1984) have shown that the functional connections between the hemispheres are not all present at hatching - the right hyperstriatum receives roughly equal numbers of projections from both the left and right thalamus, whereas the left hyperstriatum receives only 10% of the projections from right thalamic nuclei and 90% from the ipsilateral thalamus. This asymmetry is abolished from day 14 post - hatch, so that new contacts are possibly forming during the first two weeks after hatching. Perhaps, for this reason increases in spine density are peculiar to the sensitive periods of development. The formation of the remaining functional contacts soon after birth or hatching may be determined to a large extent by experience, such as imprinting or passive avoidance training in the young chick (although maturational effects cannot be disregarded). The increases in spine density and changes in spine shape observed on multipolar, *projection* neurons may therefore be relevant in this context.

However, no significant changes in the numbers of synapses in the IMHV were found (Stewart et al, 1984), which might appear to run contrary to the increases in spine density observed here, and to the arguments presented above. But, an essential difference between the present study and the electron microscopic

investigation is that the latter did not differentiate between the neuronal classes to which the synapses belonged, and thus observed total overall synapse numbers after passive avoidance training, whereas here differences in spine number were analysed only in one specific class of neuron. Also, an increase in spine number does not necessarily indicate an increase in synapse number - the latter would remain unchanged if shaft synapses are converted into spine synapses after training (see section 2.4, FIG. 4a). This possibility has been discussed in the introduction where the same theory of spine induction is applied to explain the normal increase in spine and synapse density during development (Miller and Peters, 1981; Cotman et al, 1973; Mates and Lund, 1977; Chang and Greenough, 1984). Chang and Greenough (1984) also advocate this explanation for the increases in the number of spine and shaft synapses which they found on hippocampal neurons after LTP producing tetanic stimulation. Therefore, the results of the electron microscopic investigation (Stewart et al, 1984) and the light microscopic studies of the IMHV reported here are not necessarily incompatible, but further studies are obviously needed to verify the connectionist interpretation of the results.

6.6. SELECTIVE STABILISATION

It is evident from the arguments presented in chapter 2 that a large number of synapses and spines are rapidly formed during the early postnatal periods in various animal species (see chapter 2). There is a subsequent decline in synaptic and spine density with age, which can be due to many factors, including the loss or retraction of the synaptic connections. The most prominent theory regarding this age-dependent change in synaptic number, is that espoused by Changeaux and Danchin (1977) who refer to the process as "selective stabilization" and Greenough (1978) who calls it "selective preservation". These authors argue that interneuronal

connections are initially overproduced during the early stages of development, and as a result of learning or sensory stimulation selected connections are stabilized while others are retracted. The selection process may be controlled by the activity of the neurons as a result of increased sensory experience (Changeaux and Danchin, 1977), perhaps as a means of organising the neurons into a functional network that can later control behaviour with more efficiency.

This interaction between ontogenetic factors and postnatal experience which governs changes in spine density has recently been demonstrated in several studies, especially in relation to the sensitive periods of development. For example, Rehn et al (1988) exposed rats to either deodourised air, or cyclohexanone vapour for 3 or 7 weeks from postnatal day 1, and examined the olfactory bulb. They found that although the spine density on granule cells in the olfactory bulb from all groups had increased developmentally up to day 21 with a subsequent decline to 8 weeks, the deodourised air exposed rats had a reduced spine density on granule cells from both the medial and lateral sides of the olfactory bulb, throughout this developmental period, compared to controls. The cyclohexanone exposed rats only showed a spine density decline in the lateral side of the bulb. A similar effect is observed in ferrets exposed from birth in a restricted olfactory environment saturated with geraniol odour. In this case, however, the normal decline in spine density of granule cells in the olfactory bulb that occurs from 60 days of age after the sensitive period of food/prey odour imprinting (which is at 30 to 60 days postnatal), is significantly enhanced in those ferrets reared in the restricted olfactory environment, compared to controls (Apfelbach and Weiler, 1985).

Perhaps, then, such a process is occurring on LMPNs as a result of passive avoidance training. That is, as shown in FIG. 15, there is a large increase in spine

density on all branch orders of the left hemisphere and on the lower orders of the right hemisphere as a result of training. This could represent an overproduction of spines, which normally occurs in the young animal, followed by a subsequent loss of synapses and spines, through the process of selective stabilization of the relevant connections that are involved in the storage of the memory trace. If this is the case, then some time after training we would expect to see a decrease in spine density. Although there is no evidence for this from the present data, a similar phenomenon has been observed after auditory imprinting (Wallhauser and Scheich, 1987). Wallhauser and Scheich (1987) have observed a decrease in spine density on a type of large neostriatal neuron 5 days after imprinting. They did not however, examine spine densities 24h after auditory imprinting and neither did they make a correction for those spines which could not be seen because of the opacity of the Golgi-impregnated dendrite shaft. A major problem with this study is that Wallhauser and Scheich kept the control chicks in isolation for 7 days from hatching, and some of these chicks died during the course of the experiment (Wallhauser, personal communication). Thus, it could be argued that this isolation stress has resulted in an increase in spine density in the isolated group of chicks relative to the imprinted group. Therefore, what is observed may not be a decrease in spine density due to imprinting, but an increase due to isolation stress.

However, in the study by Scheich's group, chicks were imprinted in the first *two days* post-hatch, and the visible spine densities on large multipolar neurons (similar to the ones analysed in this study) were measured when the chicks were seven days old. Although the region investigated was the neostriatum and not the IMHV, and the neurons were possibly not of the projecting type, Wallhauser and Scheich observed a drop in spine density (uncorrected for hidden spines) from a

maximum level of ~ 2.3 spines per μm down to a maximum level of ~ 1.8 , five days after auditory imprinting (and about three days *after* the sensitive period of imprinting in the chick was over). In the investigation reported here, chicks were trained when they were *one day old*, and the projection neurons were analysed 24-26h later (during the chicks' sensitive period of development), revealing increases in spine density from a maximum level of ~ 1.8 spines per μm (corrected for hidden spines) to about 2.5 spines per μm in both hemispheres. It is evident, therefore, that the spine density increases to a plateau of about 2.5 spines per μm during the sensitive period of development in the chick, after intense learning events such as passive avoidance training (and perhaps imprinting), and reverts to control levels (of ~ 1.8 spines per μm) once this period is over or after the memory storage processes are completed (FIG. 29). It could be argued, however, that whilst intense learning events such as passive avoidance training may cause increases in spine density, retraction of unneeded synapses (and therefore of spines) may be peculiar to the process of acoustic imprinting. Such arguments can only be resolved by direct experimental testing of the above hypothesis. An investigation into changes in spine density 25h after acoustic imprinting is therefore needed and spine density changes five days after passive avoidance training need to be examined, but various methodological problems have to be overcome in relation to attempting to use passive avoidance training at this time, the major one being "isolation stress".

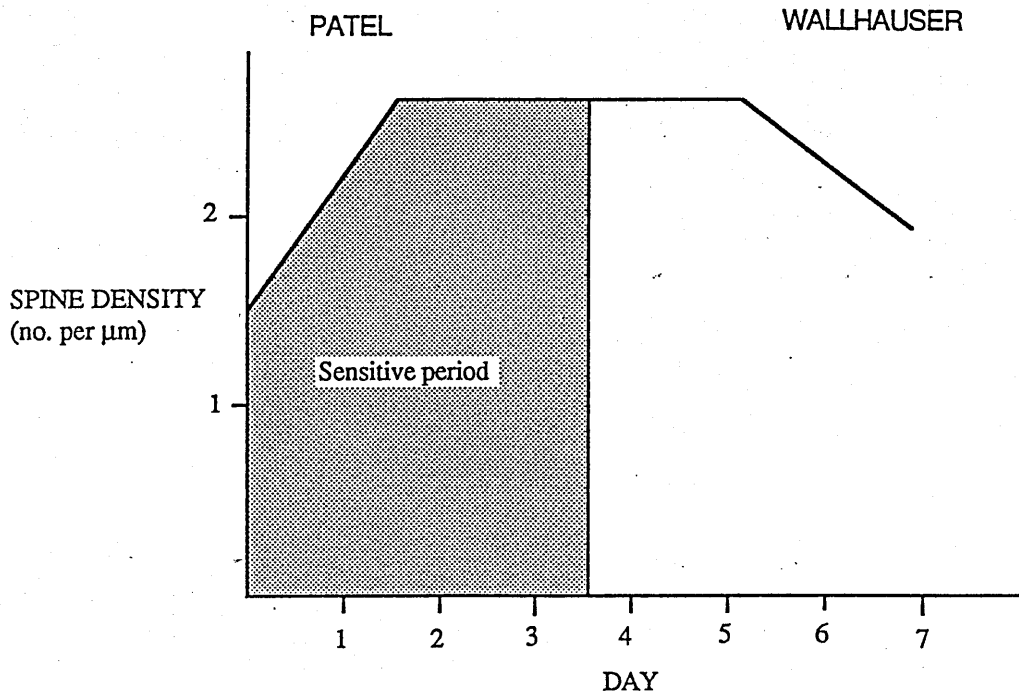


FIG. 29. The selective stabilisation hypothesis working in the chick which correlates the results from two studies: Patel (reported in this thesis) on passive avoidance training and the Wallhauser and Scheich (1987) study on auditory imprinting (see text). Intense learning experiences during the sensitive period of development will result in an over - production of spines, some of which will later be retracted or lost. Hence a drop in spine density will be observed some time after the sensitive period in the chick is over , as shown.

6.7. SOME SPECULATIONS ON THE MECHANISMS OF THE SPINE SHAPE CHANGES .

Fifkova et al (1982) and others (see chapters 1 and 2) have shown that spine shape can alter upon tetanising electrical stimulation of afferent pathways in the hippocampus, which results in an increase in spine volume. They also demonstrate that protein synthesis may be necessary for changing spine shape, because anisomycin prevents these alterations in the shape of spines after electrical stimulation. The experiments in this thesis have also revealed changes in spine shape, 25h after passive avoidance training. The question of interest, then, is what are the underlying mechanisms responsible for the observed changes in spine shape? It is likely that stimulation from a critical number of fibres or the repetitive stimulation from a small number of fibres can result in alterations in spine shape and indeed in spine density. The activation of second messenger systems may therefore be involved in this process (Mattson, 1988).

6.7.1. SPINE STRUCTURE (FIG. 30)

The application of high energy transmission electron microscopy (Wilson et al, 1983) and freeze-etching techniques (Landis and Reese, 1983) have revealed the complete organization of the microfilament matrix constituting the spine cytoskeleton. Landis and Reese (1983) have shown that three principle filaments make up the cytoskeleton of Purkinje cell spines, the largest and longest (8-10nm in diameter) exhibiting the appearance of actin. Immunocytochemical studies have confirmed the presence of actin in the spine cytoplasm (Fifkova and delay, 1982; Katsumaru et al, 1982; LeBeaux and Willemot, 1975). The second type of filament, 5-7nm in diameter is thought to be tau-protein seen in both conventional and freeze-etched electron micrographs (Landis and Reese, 1983; Coss and Perkel, 1985). Tau

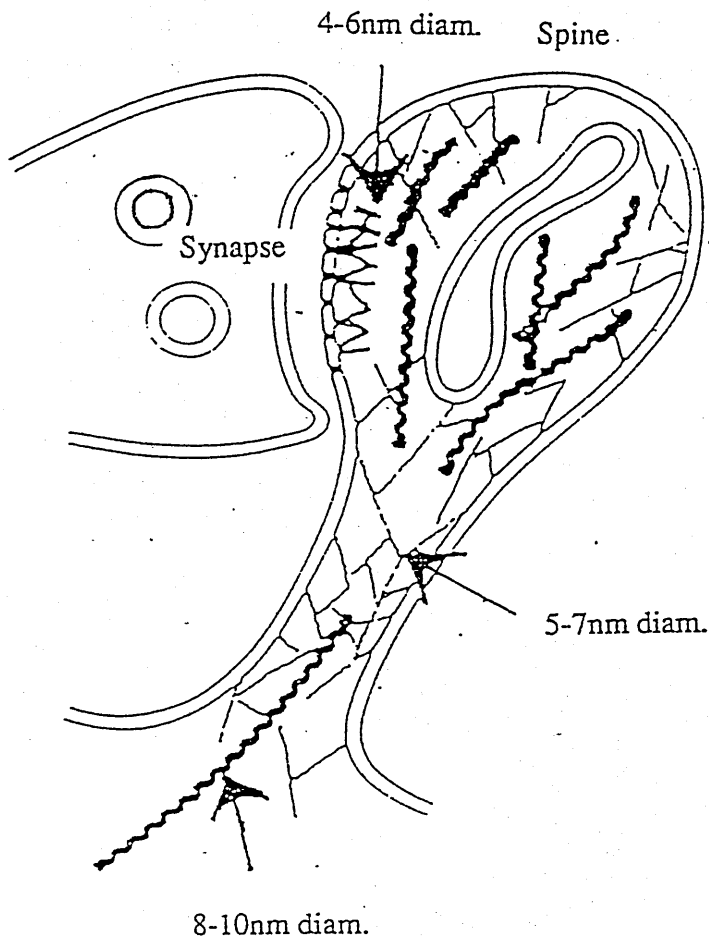


FIG. 30. Diagram of a Purkinje cell spine showing its ultrastructure (from Landis and Reese, 1983). Three main filaments have been identified by freeze-fracture studies, which are thought to be actin (8-10 nm diameter), tau proteins (5-7 nm diam.) and some 4-6 nm diameter filaments whose nature is unknown, although they may be tubulin. Together these filaments can control the structure of the spine and change its shape upon afferent stimulation as discussed in the text.

proteins, together with MAP-2 proteins, are major constituents of microtubule-associated proteins (MAPs), and are found distributed throughout the spine cytoplasm (Caceres et al, 1983). MAP-2 binds with actin to form actin filaments with numerous granulated nodules similar to those seen in freeze-etched preparations (Landis and Reese, 1983). MAPs are also important for microtubule assembly (Yamamoto et al, 1985), and for cross-linking actin filaments to form high viscosity gels.

The third type of filament, 4-6nm in diameter, found juxtaposed with intramembrane particles at the postsynaptic junction, probably corresponding to the postsynaptic thickening, may comprise α and β tubulin (Caceres et al, 1983). In combination, tubulin, actin, and MAPs are known to produce high viscosity gels *in vitro*, and actin polymers can size and self assemble into loose cross-linking networks (Griffith and Pollard, 1982) as well as interact with a wide number of micromolecules, suggesting their involvement in maintaining the rigid shape of spines.

In view of actin's cytomuscular properties. Crick (1982) and Robinson and Koch (1984) have proposed that the spine stem could be shortened and widened by the contractile actions of actin in response to bouts of depolarization. This brief contraction or "twitch" could alter the biophysical properties of the spine, such that the synaptic "weight" can be changed temporarily, thereby storing a form of 'ultra-short' memory in a Hebbian-like manner. Although this is an interesting idea (discussed at greater length above), there is no evidence that structures as small as spines actually twitch within the short time suggested by Crick.

6.7.2. SECOND MESSENGER INVOLVEMENT

One of the mechanisms by which electrical activity affects neuronal cytoarchitecture is by way of the release of neurotransmitters which then leads to changes in the postsynaptic regions through the activation of second messenger systems (Mattson, 1988). Mattson (1988) has reviewed a number of studies implicating calcium as a key regulator of neuronal cytoarchitecture. There is a substantial correlation between the influx of calcium and the effects of the neurotransmitters on cessation of neurite outgrowth, for example by serotonin and glutamate. Calcium channel blockers prevented the reduction in dendrite length normally seen in response to glutamate in cultured cells, while the calcium ionophore A23187, mimicked the effects of glutamate on dendritic outgrowth. These results have been observed in a number of cell lines: *Helisoma* cells, rat CNS neuroblastoma cells, chick dorsal root ganglion cells, and including tumour cell lines (see Mattson, 1988). Further, the effects of calcium are quite specific: under the appropriate conditions of calcium influx neurites elongate at an accelerated rate in the absence of detectable growth cone motility (Mattson, 1988). These and other results indicate that actin-based growth cone motility and microtubule-based neurite elongation can be differentially regulated by calcium.

Neurotransmitters acting via calcium may also serve important functions in the regulation of adult plasticity. The involvement of calcium in LTP in the hippocampus is well documented. For example, LTP is blocked under low calcium conditions (Dunwiddie et al, 1978); uptake and retention of calcium is increased following LTP (Baimbridge and Miller, 1981) and a large increase in dendritic calcium is associated with LTP (Kuhnt et al, 1985). Lynch and Baudry (1984; and Lynch, 1986) have presented a hypothesis suggesting that glutamate-induced calcium activity underlies LTP in the hippocampus. Their cellular model of memory

storage involves degradation of cytoskeletal fodrin by calcium-activated proteases (calpains) and a subsequent unmasking of glutamate receptors which result in a potentiation of responses to further inputs. Subsequent high frequency stimulation can then be expected to produce a larger influx of calcium because of the greater number of receptors. This will result in a further activation of calcium-dependent proteinases, which are thought to act on MAPs, fodrin (which is found in postsynaptic densities) and spectrin (which links transmembrane proteins with the actin network of the spine cytoskeleton), ultimately leading to changes in the ultrastructure of the spines.

In addition to calcium, Mattson (1988) also reviews a large number of studies suggesting the involvement of Cyclic AMP (cAMP) and inositol phospholipids (hydrolysed to protein kinase C (PKC) and IP3) in the biological actions, including the regulation of neuronal outgrowth and synaptogenesis, of several primary signals such as hormones, growth factors and neurotransmitters (Kupferman, 1980; Rasmussen, 1981; Schubert et al, 1978), in different neuron types. This evidence will not be discussed here, except to point out the relevance of these second messenger systems in adult plasticity and learning and memory processes as shown by studies on the various models such as in *Aplysia* where cAMP mediates serotonin-induced long term sensitization (Kandel and Schwartz, 1982), and in the hippocampus in which PKC is involved in LTP induction (Routtenberg, 1986).

6.7.3. POSSIBLE MECHANISMS OF ACTION OF THE SECOND MESSENGERS

One possible mechanism of action of these second messengers in inducing changes in the neuronal cytoarchitecture is that proposed by Lynch and Baudry (1984)

described briefly above. Other mechanisms are presented in summary form in FIG. 31. The effects of the second messengers are likely to focus on ionic channels and cytoskeletal elements (microtubules, microfilaments, and associated proteins, such as MAPs), and thereby not only regulate the development of the neuron from the embryonic stage but also affect its structure in the postnatal stages especially in response to learning and memory processes. Thus the changes in the number and structure of dendritic spines discussed in detail above which appears to be affected by electrical activity and the influences of endogenous factors such as opioids (Hauser et al, 1987) and phosphatidylserine (cf. Nunzi et al, 1987), can be explained by considering the actions of the various second messenger systems which act upon the protein kinases (see FIG. 31).

The second messenger kinases acting via MAP2, can influence the polymerization states of microtubules and microfilaments (Goldenring et al, 1986; Rasmussen, 1981; Matus, 1988). For example, Yamamoto et al (1985) show that cyclic AMP dependent and Ca^{2+} , calmodulin-dependent protein kinases can inhibit microtubule assembly by phosphorylating MAPs (particularly MAP2), tau proteins, and tubulin. The Ca^{2+} , calmodulin system also appears to be more effective in this regulatory process than the cAMP system. The phosphorylation state of MAP2 also determines the extent of actin filament cross-linking activity (Goldenring et al, 1986). Unphosphorylated MAP2 bundles actin filaments, whereas the phosphorylated molecule still binds the filaments, but they are no longer bundled (Sattilaro, 1986). Such actions can explain the effects (briefly discussed above) of these second messenger systems on neurite elongation, and growth cone motility. The involvement of protein kinases (e.g. PKC) in synaptic plasticity has been demonstrated by Routtenberg (1984) for the maintenance of LTP in the hippocampus and by Ali et al (in preparation) in memory storage processes in the

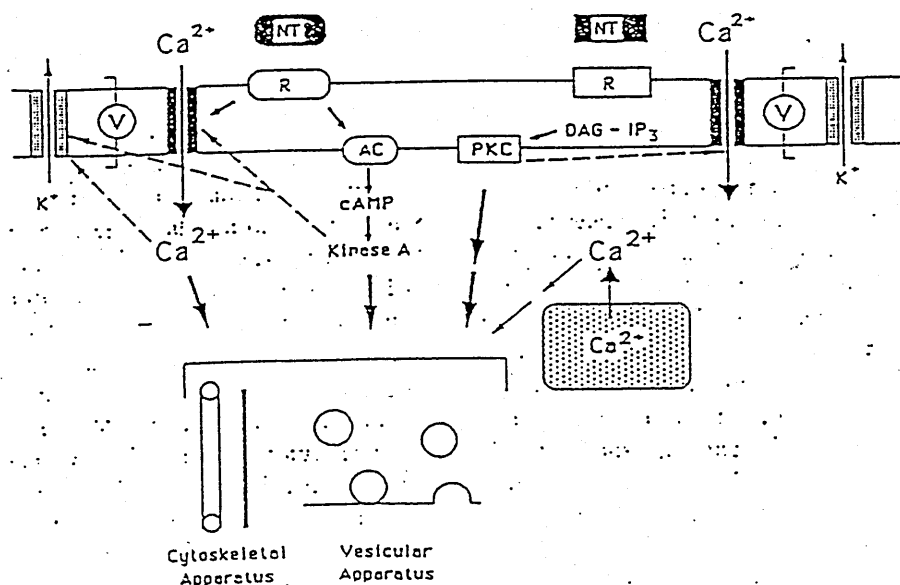


FIG. 31. The actions of the various second messengers in altering spine shape (see text). Intracellular calcium can be elevated by NT-stimulated influx through either *receptor-coupled or voltage-sensitive* plasma membrane channels; GABA and other inhibitory neurotransmitters can prevent the effects of excitatory transmitters on calcium influx through voltage dependent channels. Cyclic AMP increases upon stimulation of receptor-coupled adenylate cyclase and then activates kinase A. The receptor-mediated hydrolysis of inositol phospholipid results in the liberation of diacylglycerol (DAG) which activates PKC, and IP₃ which mobilises calcium from intracellular stores. The activated kinase A and PKC can regulate calcium influx by acting directly on calcium channels or indirectly by altering membrane potential (Kaczmarek et al, 1986; Kostyuk, 1986; Lemos et al, 1986). The calcium can act directly or through a kinase or calmodulin to regulate the behaviours of the cytoskeletal elements in altering neurite elongation, growth cone motility and in the postnatal stages spine and dendrite morphology.

chick. Ali et al show that inhibitors of PKC (such as melittin) when injected 5 min before or 5 min after training, into the forebrain (in the region of the IMHV) causes amnesia for the passive avoidance training task.

Coss and Perkel (1985) and Fifkova and van Harreveld (1977) further suggest a mechanism for altering spine shape (and dendrite morphology) that involves such phosphorylation / dephosphorylation of MAPs, resulting in actin filament and microtubule disassembly / assembly, respectively. Transient current flow generated by ion influx could produce an electrosmotic flow of extracellular water into the postsynaptic region, resulting in an increase in the volume of the spine to a certain extent (the cytoskeletal lattice restraining further spine head swelling). The insertion of new plasma membrane could then subsequently stabilise the change in spine shape for which protein synthesis will be necessary. Indeed Fifkova et al (1982) show that anisomycin prevents the shape changes seen 4 min after tetanic stimulation of the perforant path to the hippocampus. Further, polyribosomal aggregates have also been observed at the postsynaptic region in the spine head and spine stem suggesting that the synthesis of proteins could occur at the sites where it would be used. Such mechanisms may well be operating in the IMHV following passive avoidance training, thereby giving rise to the observed spine shape changes.

6.8. CONCLUSIONS

The amnesia experiments clearly demonstrate the involvement of dendritic spines in the storage of information related to the passive avoidance training task. To my knowledge this is the first demonstration of an increase in spine density within 25h of a single-trial passive avoidance learning task in the chick or in any other animal.

This has important consequences in consideration of the role and function of dendritic spines in learning and memory and also for inferences regarding its function. The large increases in spine density may imply an increase in synapse density only on LMPNs of the IMHV after training. Alternatively, the number of synapses on the LMPNs may remain the same, but a majority of the shaft synapses may be transformed into spine synapses, thus giving an increase in spine density without a change in synapse number. Both possibilities are equally likely, and therefore further experimentation is needed to decide between the two. In either case, the biophysical interpretation from computer simulation studies (in other laboratories) of the spine shape changes, together with the enhanced spine density recorded here, indicates that these changes confer a greater information processing capacity in the left hemisphere more so than in the right, as a consequence of passive avoidance learning.

These results are also important for interpreting the correlation between low spine density (including abnormal spine shapes) and mental disorders and dementia in humans, which involve losses in learning and memory (discussed in the introduction). According to the "connectionist" theory of dendritic spines (which states that spines are formed in order to make synaptic connections with axons that are distant from the dendrite shaft), it could be argued that in dementia patients synaptic contacts are lost, resulting in a retraction or loss of the dendritic spines. This loss of the number of spines on the dendrite shaft will have marked effects on the information processing capacity of the dendritic tree as well as the neuronal network to which that neuron may be a part, as demonstrated by computer simulation studies (see introduction), ultimately resulting in behavioural dysfunction. Another possibility is that these disorders have a profound effect on the plasticity of the nervous system, such that the capacity for morphological

changes in dendrites and dendritic spines is lost. This would ultimately have an adverse effect on the processing and storage capabilities of the brain. In any case, the results reported in this thesis have drawn us one step closer to understanding the neural basis of information storage in the vertebrate, and eventually also, in the human brain.

CHAPTER 7

FUTURE DIRECTIONS

There are several more experiments that need to be carried out in order to test several hypotheses which have emerged from the investigations reported in this thesis. Some of these have been mentioned in the various chapters, and are briefly re-iterated here. Firstly the alterations in dendritic spine number and shape needs to be correlated with other ultra-structural observations such as

(1) Whether each dendritic spine on the LMPNs from the trained and control cells possess at least one synaptic contact, and which types they may be. That is, gray type 1 synapses, which are asymmetric with round vesicles at the presynaptic area; or type 2 synapses, which have symmetric dense projections and pleomorphic vesicles, and which are thought to be inhibitory.

If each spine on LMPNs from both trained and control chicks has at least one synapse, particularly on the spine head, then it would provide some support for the "synaptic induction" hypothesis of dendritic spine formation presented in the introduction: spines may be formed by a trophic effect of a synaptic contact on the dendrite shaft, such that the dendritic membrane appears to be 'pulled out', to produce a sessile spine (FIG. 4). A developmental study would be needed in order to test this hypothesis.

(2) If there is no significant difference between the total number of synapses on the LMPNs from the trained and control chicks, but there is a change in distribution of the synapses, such that there are more synapses on the spine heads, rather than on

the dendrite shafts in the trained chicks compared to the controls, then the "synaptic induction" hypothesis would be further supported.

If some of the spines appear to be synapse-free, then the growth of dendritic spines could be similar to that found in the cerebellum, where spines seem to grow in the absence of any synaptic connection.

(3) Although changes in the spine head diameter and spine stem length have been measured in Golgi-impregnated tissue, alterations in the diameter of the stem have not. From the discussion on the function of dendritic spines (chapter 2), it is evident that changes in spine stem diameter could have a profound influence on the physiological characteristics of the spine. Thus, if the spine stem has become thicker as a result of training, then the efficacy of a synaptic contact on the spine head is probably altered. Therefore, a determination of changes in the thickness of the spine stems on LMPNs is required.

In order to test the above hypotheses, quantitative electron microscopic measurements need to be made. These electron microscope measurements can be made on actual LMPNs from the trained and control chicks, by the use of the Golgi-gold toned method devised by Fairen et al (1977) (see appendix C, for details of the procedure). With this method the tissue is initially stained by the rapid Golgi technique described in chapter 3, which enables the experimenter to identify LMPNs at the light microscope level. It is impractical to thin-section Golgi-stained tissue for electron microscopy, because either the silver chromate precipitate will "fall out" of the thin section due to its weight, or it will appear as a dense mass that covers the whole of the inside of the cell. Thus, the tissue sections are gold-toned according to the protocol given below (appendix C), which has the effect of

displacing the silver chromate precipitate from the Golgi-impregnated neurons, with gold particles. The tissue is then serially sectioned at the silver interference level and lifted off the water surface with slot grids that are coated with formvar, or pioloform. The grids are stained with Reynolds lead and uranyl acetate (4%, aq.) for electron microscopy. The gold remains in the cells as small particles which appear as small dense spots on the electron micrograph of the thin sectioned Golgi-gold toned tissue (FIG. 32). An example of a micrograph of a Golgi-gold toned section is given in FIG. 32, which is a photograph of a section through a Gold-toned neuron (FIG. 6, bottom photograph), which was identified by light microscopy as being a large, multipolar, projection neuron. A light microscopic photograph of neurons after gold toning is given in FIG. 6. Unfortunately, the tissue appears not to be well fixed, and because the whole procedure takes a very long time, further experiments were not possible within the time limits of the studentship. However, some spines can still be identified on the dendrite shaft and which can be followed through adjacent sections, although the poor fixation makes identification of different synaptic types difficult. The sections in this case were obtained by taking serial thin cross-sections of the cell shown in FIG. 6 (bottom photograph). The depth of cut along the cell is also fairly easy to follow because the cell can still be visible through the resin block under the light microscope. FIG. 32 is a micrograph of a section taken near the cell body of the gold toned neuron in the bottom picture of FIG. 6. Some spines can be seen projecting from the dendrite shaft in FIG. 32.

With well fixed tissue and careful sectioning, good useable preparations are possible, and some measurements can be made on the photomicrographs of: spine stem width; the identification of synaptic types located on the spines and dendrite

FIG. 32. Micrograph of the Golgi-gold toned neuron shown in FIG. 6 (bottom photograph) serially sectioned with the microtome at approximately 50-60 nm. This thin section was taken near the cell body, and shows a 1st order dendrite with spines emanating from the shaft. Unfortunately, the fixation is not very good, so the ultrastructure of the synapses cannot be clearly seen.

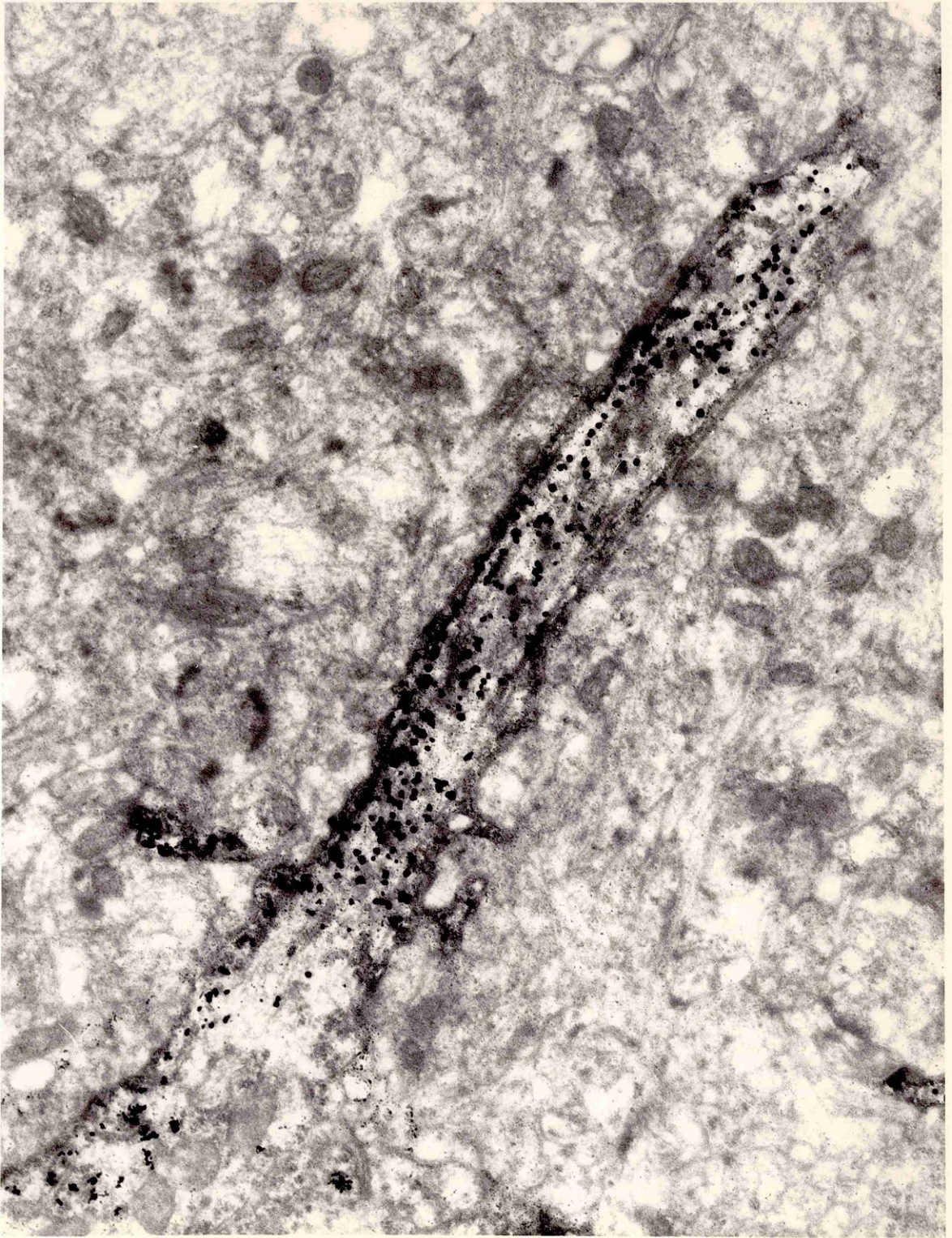


FIG.32

shafts, from which the ratio of shaft to spine synapses can be estimated; and spines without any synaptic contacts can be found. Of course, some tissue sections will be lost, even by taking serial sections, but this should not affect the actual measurements substantially.

(4) Further light microscopic investigations need to be performed in order to assess firstly the time course of the change in spine density: is there a significant increase in spine density at an earlier time point, before 24h after training? The studies of Chang and Greenough (1984) on hippocampal LTP suggests that spines can form within 15 min of direct electrical stimulation of the neurons.

(5) Do the spine shape changes occur as early as 9 or 10 min after training, in correlation with results from honeybee one-trial place learning (see introduction)? If so, are they also found in chicks which either fail to learn the task or are rendered amnesic?

(6) The effect of electroshock per se on spine dimensions in the resting brain needs to be established as mentioned on page 84.

(7) A similar morphological study of changes in spines and dendrites in the LPO and the PA, is needed because these areas are also involved in the learning of the passive avoidance task, and the LPO also shows an increase in the volume density of synapses 24h after training.

(8) The involvement of protein synthesis in the development of dendritic spines could be directly tested by the injection of an amnesic dose of either glutamate or

antibiotics such as anisomycin, CXM, or emetine into the IMHV, and morphological measurements performed 24h after training.

(9) It is important to know if the spine density increases observed 24h after training are followed by a decrease a few days later. This would test the selective stabilisation hypothesis discussed in the thesis. However, several practical problems need to be overcome, the primary one being that of leaving the chicks in a controlled environment for more than a day or two. Isolation stress, or the effects of imprinting could confound the results.

REFERENCES

- Abrams, T.W. (1985). Activity-dependent presynaptic facilitation: An associative mechanism in *Aplysia*. *Cell Mol. Neurobiol.* 5(1-2): 123-145.
- Abraham, W.C. and Goddard, G.V. (1984). Functions of afferent coactivity in long-term potentiation. In *Neurobiology of learning and memory*. Eds: G. Lynch, J.L. McGaugh and N.M. Weinberger.
- Abrams, T.W. and Kandel, E.R. (1988). Is contiguity detection in classical conditioning a system or a cellular property? learning in *Aplysia* suggests a possible molecular site. *TINS*. 11(4): 128-135.
- Aghajanian, G.K. and Bloom, F.E. (1967). The formation of synaptic junctions in developing rat brain: a quantitative electron microscopic study. *Brain Res.* 6: 716-727.
- Agranoff, B.W., Burrell, H.R., Dokas, L.A. and Springer, A.D. (1976). Progress in biochemical approaches to learning and memory. In *Psychopharmacology: a generation of progress*, Eds: M.A. Lipton, A. Dimasco and K.F. Killam. pp 623-35. Raven: NY.
- Aleksidze, N., Potempska, A., Murphy, S., and Rose, S.P.R. (1981). passive avoidance in the young chick affects forebrain α -bungarotoxin and serotonin binding. *Abstr. 8th ISN.*:382.
- Alkon, D.L. (1974). Associative training of *Hermissenda*. *J. Gen. Physiol.* 64: 70-84.
- Andrew, R.J. (1983). Lateralisation of emotional and cognitive function in higher vertebrates with special reference to domestic chicks. In *Advances in vertebrate neuroethology*, Eds: J.P. Ewert, R.R. Capranica, and D.J. Ingle. Plenum: London. pp 447-510.
- Andrew, R.J. and Brennan, A. (1983). The lateralization of fear behaviour in the male domestic chick: A developmental study. *Anim. Behav.* 31: 1166-1176.
- Andrew, R.J., Mench, J. and Rainey, C. (1980). Right-left asymmetry of response to visual stimuli in the domestic chick. In *Advances in the analysis of visual behaviour* Eds: D.J. Ingle, M.A. Goodale and R.J.W. Mansfield. MIT press.

- Apfelbach, R. and Weiler, E. (1985). Olfactory deprivation enhances normal spine loss in the olfactory bulb of developing ferrets. *Neurosci. Letts.* 62: 169-173.
- Bailey, C.H. and Chen, M. (1983). Morphological basis of long-term habituation and sensitization in *Aplysia*. *Science*. 270: 91-93.
- Baimbridge, K.G. and Miller, T.J. (1981). Calcium uptake and retention during long-term potentiation of neuronal activity in the rat hippocampal slice preparation. *Brain Res.* 221: 299-305.
- Baloyannis, S.J., Karakatsanis, K., Karathanasis, J., Apostolakis, M. and Diacoyannis, A. (1983). Effects of GABA, glycine and sodium barbiturate on dendritic growth in vitro. *Acta. Neuropathol. (Berl.)*. 59: 171-182.
- Barlow, H.B. (1975). Visual experience and cortical development. *Nature*. 258: 199-204.
- Bartlett, W.P. and Banker, G.A. (1984). An electron microscope study of the development of axons and dendrites by hippocampus neurons in culture. II. Synaptic relationships. *J. Neurosci.* 4: 1954-1965.
- Berard, D.R., Burgess, J.W. and Coss, R.G. (1981). Plasticity of dendritic spine formation: a state dependent stochastic process. *Int. J. Neurosci.* 13: 93-98.
- Berkley, J.H. (1897). Studies on the lesions produced by the action of certain poisons on the cortical nerve cell. *Johns Hopkins Hospital Reports*, 6: 1-93.
- Berry, M. and Bradley, P. (1976a). the growth of the dendritic trees of Purkinje cells in irradiated agranular cerebellar cortex. *Brain Res.* 116: 361-387.
- Berry, M. and Bradley, P. (1976b). The growth of dendritic trees of Purkinje cells in the cerebellum of the rat. *Brain Res.* 112: 1-35.
- Berry, M. and Flinn, R.M. (1984). Vertex analysis of the Purkinje cell dendritic trees in the cerebellum of the rat. *Proc. R. Soc. Lond. B.* 221: 321-348.
- Berry, M., Bradley, P. and Borges, S. (1978). Environmental and genetic determinants of connectivity in the central nervous system - an approach through dendritic field analysis. *Prog. Brain Res.* 48: 133-148.

- Berry, M., Sadler, M. and Flinn, R. (1986). Vertex analysis of neural tree structures containing trichotomous nodes. *J. Neurosci. methods.* 18: 167-177.
- Berry, M., Sievers, J. and Baumgarten, H.G. (1980). The influence of afferent fibres on the development of the cerebellum. In *A multidisciplinary approach to brain development*. Eds: C. diBenedetta, R. Balasz, G. Gambos and G. Porcellati. North-Holland Biomed. Press: Amsterdam.
- Black, I.B., Adler, J.A., Dreyfus, C.F. Friedman, W.F., LaGamma, E.F. and Roach, A.H. (1987). Biochemistry of information storage in the nervous system. *Science* 236: 1263-1268.
- Black, J.E., Jones, A.L., Anderson, B.J., Isaacs, K.R., Alcantara, A.A. and Greenough, W.T. (1987). Cerebellar plasticity: preliminary evidence that learning rather than repetitive motor exercise alters cerebellar cortical thickness in middle-aged rats. *Soc. for Neurosci. Abstr.* 13(3): 1596.
- Bliss, T.V.P. (1979). Synaptic plasticity in the hippocampus. *TINS.* 2:42-45.
- Bliss, T.V.P. and Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetised rabbit following stimulation of the perforant path. *J. Physiol. (London)* 232: 331-356.
- Bliss, T.V.P., Chung, S.-H. and Stirling, R.V. (1974). Structural and functional development of the mossy fibre system in the hippocampus of the postnatal rat. *J. Physiol. (London)* 239: 92-93P.
- Boothe, R.G., Greenough, W.T., Lund, J.S. and Wrege, K. (1979). A quantitative investigation of spine and dendrite development of neurons in visual cortex (area 17) of *macaca nemestrina* monkeys. *J. Comp. Neurol.* 185: 473-490.
- Bowman, R.E., and Kottler, P.D. (1970). In *Biochemistry of brain and behaviour*, Eds: R.E. Bowman, S.P. Datta. pp 301-326. Plenum: NY.
- Bradley, P (1985). A light and electron microscopic study of the development of two regions of the chick forebrain. *Dev. Brain Res.* 20: 83-88.
- Bradley, P. and Berry, M. (1976a). The effects of reduced climbing fibre and parallel fibre input on Purkinje cell dendritic growth. *Brain. Res.* 109: 133-

- Bradley, P. and Berry, M. (1976b). Quantitative effects of climbing fibre deafferentation on the adult Purkinje cell dendritic tree. *Brain Res.* 112: 133-140.
- Bradley, P. and Berry, M. (1978). Development of Purkinje cells in the murine mutants 'weaver' and 'staggerer'. *Brain Res.* 142: 135-141.
- Bradley, P. and Horn, G. (1979). Neuronal plasticity in the chick brain: morphological effects of visual experience on neurons in the hyperstriatum accessorium. *Brain Res.* 162: 148-153.
- Bradley, P., Horn, G. and Bateson, P. (1981). Imprinting: An electron microscopic study of chick hyperstriatum ventrale. *Exp. Brain Res.* 4 : 115-120.
- Bradshaw, J.L. and Nettleton, N.C. (1981). The nature of hemispheric specialization in man. *The Behavioural and Brain Sciences.* 4: 51-92.
- Brandon, J.G. and Coss, R.G. (1982). Rapid dendritic spine stem shortening during one-trial learning : the honeybee's first orientation flight. *Brain Res.* 252: 51-61.
- Brown, M.W. and Horn, G. (1979). neuronal plasticity in the chick brain: electrophysiological effects of visual experience on hyperstriatal neurons. *Brain Res.* 162: 142-147.
- Burgess, J.W. and Coss, R.G. (1980). Crowded jewel fish show changes in dendritic spine density and spine morphology. *Neurosci. Letts.* 17: 277-281.
- Burgess, J.W. and Coss, R.G. (1983). Rapid effect of biologically relevant stimulation on tectal neurons: changes in dendritic spine morphology after nine minutes are retained for 25h. *Brain Res.* 266: 217-223.
- Burgoyne, R.D. and Rose, S.P.R. (1981). Subcellular localization of increased incorporation of ³H-fucose following passive avoidance learning in chicks. *Neurosci. Letts.* 19: 343-348.
- Caceres, A. and Steward, O. (1983). Dendritic reorganisation in the denervated dentate gyrus of the rat following entorhinal cortical lesions: A Golgi and

- electron microscope analysis. *J. Comp. Neurol.* 214: 387-403.
- Caceres, A., Payne, M.R., Binder, L.T. and Steward, O. (1983). Immunocytochemical localisation of actin and microtubule associated protein MAP-2 in dendritic spines. *Proc. Nat. Acad. Sci. USA.* 80: 1738-1742.
- Calvet, M.C., Drian, M.J. and Privat, A. (1974). Spontaneous electrical patterns in cultured Purkinje cells grown with an anti-mitotic agent. *Brain Res.* 79: 285-290.
- Calvet, M., Lepault, A. and Calvet, J. (1976). A procion yellow study of cultured Purkinje cells. *Brain Res.* 111: 399-406.
- Carew, T.J., Castelluci, V.F. and Kandel, E.R. (1971). An analysis of dishabituation and sensitization of the gill withdrawal reflex in *Aplysia*. *Int. J. Neurosci.* 2: 79-98.
- Carew, T.J., Pinsker, H. and Kandel, E.R. (1972). Long-term habituation of a defensive withdrawal reflex in *Aplysia*. *Science* 175: 451-454.
- Casamenti, F., Mantovani, P., Amaduci, L. and Pepeu, G. (1979). Effect of phosphatidylserine on acetylcholine output from the cerebral cortex of the rat. *J. Neurochem.* 32: 529-533.
- Catala, I., Ferrer, I., Galofré, E. and Fabrigues, I. (1988). Decreased numbers of dendritic spines on cortical pyramidal neurons in dementia. A quantitative Golgi study on biopsy samples. *Human Neurobiol.* 6: 255-259.
- Chang, F.-L. F. and Greenough, W.T. (1982). Lateralised effects of monocular training on dendritic branching in adult split-brain rats. *Brain Res.* 232: 283-294.
- Chang, F.-L. F. and Greenough, W.T. (1984). Transient and enduring morphological correlates of synaptic activity and efficacy change in the rat hippocampal slice. *Brain Res.* 309: 35-46.
- Changeaux, J.-P. and Danchin, A. (1977). Biochemical model for the selective stabilisation of developing synapses. In *Synapses*, Eds: G.A. Cottrell and P.N.R. Usherwood. Academic: NY.
- Changeaux, J.P. and Mikoshiba, K. (1978). Genetic and epigenetic factors regulating synapse formation in vertebrate cerebellum and neuromuscular

- junction. In *Maturation of the nervous system*. Eds: M.A. Corner, R.E. Baker, N.E. van de Poll, D.F. Swaab and H.B.M. Uylings.
- Chen, S. and Hillman, D.E. (1982). Marked reorganisation of Purkinje cell dendrites and spines in adult rat following vacating of synapses due to deafferentation. *Brain Res.* 245: 131-135.
- Cipello-Neto, J., Horn, G. and McCabe, B.J. (1982). Hemispheric asymmetry and imprinting: the effect of sequential lesions to the hyperstriatum ventrale. *Exp. Brain Res.* 48: 22-27.
- Cohan, C.S. and Kater, S.B. (1986). Suppression of neurite elongation and growth cone dynamics by electrical activity. *Science.* 232: 1638-1640.
- Coleman, P.D. and Risen, S. (1968). Environmental effects on cortical dendritic fields. 1. Rearing in the dark. *J. Anat.* 102: 363-374.
- Colonnier, M. (1968). Synaptic patterns on different cell types in the different laminae of the cat visual cortex: an electron microscopic study. *Brain Res.* 9: 268-287.
- Corwin, J., dean, R.L., Bartus, R.T., Rotrosen, J. and watkins, D.L. (1985). Behavioural effects of phosphatidylserine in the aged fischer 344 rat: Amelioration of passive avoidance deficits without changes in psychomotor task performance. *Neurobiol. Aging.* 6: 11-15.
- Coss, R.G. (1979). Delayed plasticity of an instinct: Recognition and avoidance of two facing eyes by the jewel fish. *Dev. Psychobiol.* 12: 335-345.
- Coss, R.G. and Brandon, J.G. (1982). Rapid changes in dendritic spine morphology during the honeybee's first orientation flight. In *The biology of social insects*, Eds: M.D. Breed, C.D. Michener and H.E. Evans. Westview press: Boulder. pp 338-342.
- Coss, R.G., Brandon, J.G. and Globus, A. (1980). Changes in the morphology of dendritic spines on honeybee calycal interneurons associated with cumulative nursing and foraging activities. *Brain Res.* 192: 49-59.
- Coss, R.G. and Burgess, J.W. (1981). Jewel fish retain juvenile schooling pattern after crowded development. *Dev. Psychobiol.* 14: 452-457.

- Coss, R.G., and Globus, A. (1978). Spine stems on tectal interneurons in jewel fish are shortened by social stimulation. *Science. (Wash. D.C.)* 200: 787-789.
- Coss, R.G. and Globus, A. (1979). Social experience affects the development of dendritic spines and branches on tectal interneurons in the jewel fish. *Dev. Psychobiol.* 12: 347-358.
- Coss, R.G. and Perkel, D.H. (1985). The function of dendritic spines: A review of theoretical issues. *Beh. and Neural Biol.* 44: 151-185.
- Cotman, C.W., Taylor, D. and Lynch, G. (1973). Ultrastructural changes in synapses in the dentate gyrus of the rat during development. *Brain Res.* 63: 205-213.
- Cragg, B.G. (1975a). The development of synapses in the kitten visual cortex during visual deprivation. *Exp. Neurol.* 46: 445-451.
- Cragg, B.G. (1975b). The development of synapses in the visual system of the cat. *J. Comp. Neurol.* 160: 147-166.
- Crick, F. (1982). Do dendritic spines twitch? *TINS* 5:44-46.
- Crow, T. (1988). Cellular and molecular analysis of associative learning in *Hermisenda*. *TINS* 11(4): 136-142.
- Denenberg, V.H. (1981). Hemispheric laterality in animals and the effects of early experience. *Behav. Brain. Sci.* 4: 1-9.
- Desmond, N.L. and Levy, W.B. (1983). Synaptic correlates of associative potentiation / depression: an ultrastructural study in the hippocampus. *Brain Res.* 265: 21-30.
- DeVoogd, T.J., Chang, F.-L.F., Floeter, M.K. Jencius, M.J. and Greenough, W.T. (1981). Distortions induced in neuronal quantification by camera lucida analysis: comparison using a semi-automated data acquisition system. *J. Neurosci. methods.* 3: 285-294.
- DeVoogd, T.J., Brenowitz, E.A. and Arnold, A.P. (1988). Small sex differences in song control dendrites are associated with minimal differences in song capacity. *J. Neurobiol.* 19(3): 199-209.

- DeVoogd, T.J. and Nottebohm, F. (1981a). Sexual differences in dendritic morphology of a song control nucleus in the canary: a quantitative Golgi study. *J. Comp. Neurol.* 196: 309-316.
- DeVoogd, T.J., Norden, H. and Gould, C.G. (1986). Dendritic retraction and spine elimination in females contributes to sexual dimorphisms in the avian song system. *Soc. neurosci. Abstr.* 12: 1214.
- Diamond, M.C. (1967). Extensive cortical depth measurements and neuron size increases in the cortex of environmentally enriched rats. *J. Comp. Neurol.* 131: 357-364.
- Diamond, M.C., Law, F., Rhodes, H., Lindner, B., Rosenzweig, M.R., Krech, D. and Bennett, E.L. (1966). Increases in cortical depth and glia numbers in rats subjected to enriched environments. *J. Comp. Neurol.* 128: 117-125.
- DiPrisco, G.V. (1984). Hebb synaptic plasticity. *Prog. Neurobiol.* 22: 89-102.
- Drago, F., Canonico, P.L. and Scapagnini, U. (1981). Behavioural effects of phosphatidylserine in aged rats. *Neurobiol. Aging.* 2: 209-213.
- Dunn, A.J. (1980). Neurochemistry of learning and memory: an evaluation of recent data. *Ann. Rev. Psychol.* 31: 343-390.
- Dunwiddie, T., Madison, D. and Lynch, G. (1978). Synaptic transmission is required for initiation of long-term potentiation. *Brain Res.* 150: 413-417.
- Fairén, A., Peters, A. and Saldanha, J. (1977). A new procedure for examining Golgi impregnated neurons by light and electron microscopy. *J. Neurocytol.* 6: 311-337. Feldman, M.L. and Dowd, C. (1975). Loss of dendritic spines in aging cerebral cortex. *Anat. Embryol.* 148: 179-301.
- Feldman, M.L. and Dowd, C. (1975). Loss of dendritic spines in aging cerebral cortex. *Anat. Embryol.* 148: 279-301.
- Feldman, M.L. and Peters, A. (1979). A technique for estimating the total spine number on Golgi-impregnated dendrites. *J. Comp. Neurol.* 188: 527-542.
- Ferrer, I., Fabrigues, I., Rairiz, J. and Galofré, E. (1986). Decreased numbers of dendritic spines on cortical pyramidal neurons in human chronic alcoholism. *Neurosci. Letts.* 69: 115-119.

- Fiala, B.A., Joyce, J.N. and Greenough, W.T. (1978). Environmental complexity modulates growth of granule cell dendrites in developing, but not adult hippocampus of rats. *Exp. Neurol.* 59: 372-383.
- Fifkova, F. (1968). Changes in the visual cortex of rats after unilateral deprivation. *Nature (London)*. 220: 379-381.
- Fifkova, F. (1970a). The effect of monocular deprivation on the synaptic contacts of the visual cortex. *J. Neurobiol.* 1: 285-294.
- Fifkova, F. (1970b). Changes of axosomatic synapses in the visual cortex of monocularly deprived rats. *J. Neurobiol.* 2: 61-71.
- Fifkova, F. (1970c). The effects of unilateral deprivation on visual centre in rats. *J. Comp. Neurol.* 140: 431-438.
- Fifkova, E. and Anderson, C.L. (1981). Stimulation-induced changes in dimensions of stalks of dendritic spines in the dentate molecular layer. *Exp. Neurol.* 74: 621-627.
- Fifkova, E. and Delay, R.J. (1982). Cytoplasmic actin in neuronal processes as a possible mediator of synaptic plasticity. *J. Cell. Biol.* 95(1): 345-350.
- Fifkova, E. and Van Harreveld, A. (1977). Long-lasting morphological changes in dendritic spines of dentate granule cells following stimulation of the entorhinal area. *J. Neurocytol.* 6: 211-230.
- Fifkova, E. and Van Harreveld, A. (1977). Long-lasting morphological changes in dendritic spines of dentate granule cells following stimulation of the entorhinal area. *J. Neurocytol.* 6: 211-230.
- Fifkova, E., Markham, J.A. and Delay, R.J. (1983). Calcium in the spine apparatus of dendritic spines in the dentate molecular layer. *Brain Res.* 266: 163-168.
- Fifkova, E., Anderson, C.L., Young, S.J. and Van Harreveld, A. (1982). Effect of anisomycin on stimulation induced changes in dendritic spines of the dentate granule cells. *J. Neurocytol.* 11: 183-210.
- Gaston, K.E. and Gaston, M.G. (1984). Unilateral memory after bilateral

- discrimination training: left hemisphere dominance in the chick? *Brain Res.* 303: 190-193.
- Gibbs, M.E. and Ng, K.T. (1977). Psychobiology of learning and memory: towards a model of memory formation. *Biobehav. Rev.* 1: 113-136.
- Glassman, E. (1969). The biochemistry of learning: an evaluation of the role of RNA and protein. *Ann. Rev. Biochem.* 38:605-646.
- Globus, A. and Scheibel, A.B. (1967). The effect of visual deprivation on cortical neurons: a Golgi study. *Exp. Neurol.* 19: 331-345.
- Globus, A., Rosenzweig, M.R., Bennet, E.L. and Diamond, M.C. (1973). Effects of differential experience on dendritic spine counts in rat cerebral cortex. *J. Comp. Physiol. Psy.* 82: 175-181.
- Gluschenko, T.S., Pevsner, L.Z., Bromberg, V.A. (1977). Synthesis of brain specific proteins in rat hippocampus in the course of conditioned reflex formation. *Acta. Nerva. Super.* 19: 148.
- Goldenring, J.R., Vallano, M.L., Lasher, R.S., Veda. T. and Delorenzo, R.J. (1986). Association of calmodulin-dependent kinase II and its substrate proteins with neuronal cytoskeleton. *Prog. Brain Res.* 69: 341-354.
- Gona, A.G. (1975). Golgi studies of cerebellar maturation of frog tadpoles. *Brain Res.* 95: 132-136.
- Gray, E.G. (1959). Axosomatic and axodendritic synapses of the cerebral cortex. *J. Anat.* 93: 420-433.
- Green, E.J., Greenough, W.T. and Schlumpf, B.E. (1983). Effects of complex or isolated environments on cortical dendrites of middle-aged rats. *Brain Res.* 264: 233-240.
- Greenough, W.T. (1978). Development and memory: the synaptic connection. In *Brain and learning*, Ed: T. Teyler. Greylock: Stamford, Conn.
- Greenough, W.T. (1984). Structural correlates of information storage in the mammalian brain: a review and hypothesis. *TINS.* 229-233.
- Greenough, W.T. (1986). What's special about development? Thoughts on the bases of experience - sensitive synaptic plasticity. *Dev. Neuropsychobiol.* :

387-407.

- Greenough, W.T. and Bailey, C.H. (1988). The anatomy of a memory: Convergence of results across a diversity of tests. *TINS* 11(4): 142-147.
- Greenough, W.T. and Chang, F-L. F. (1985). Synaptic structural correlates of information storage in the nervous system. In *Synaptic plasticity*. Ed: Cotman, C.W. Guilford.
- Greenough, W.T. and Volkmar, F.R. (1973). Pattern of dendritic branching in occipital cortex of rats reared in complex environments. *Exp. Neurol.* 40: 491-504.
- Greenough, W.T., Juraska, J.M. and Volkmar, F.R. (1979). Maze training effects on dendritic branching in occipital cortex of adult rats. *Beh. and Neural Biol.* 26(3): 287-297.
- Greenough, W.T., Larson, J.R. and Withers, G.S. (1985b). Effects of unilateral and bilateral training in a reaching task on dendritic branching of neurons in the rat motor-sensory forelimb cortex. *Beh. and Neural Biol.* 44: 315-324.
- Greenough, W.T., Volkmar, F.R. and Juraska, J.M. (1973). Effects of rearing complexity on dendritic branching in frontolateral and temporal cortex of the rat. *Exp. Neurol.* 41: 371-378.
- Griffith, L.M. and Pollard, T.D. (1982). The interaction of actin filaments with microtubules and MAPs. *J. Biol. Chem.* 257: 9143-9151.
- Gurney, M.E. and Konishi, M. (1980). Hormone-induced sexual differentiation of brain and behaviour in zebra finches. *Science.* 208: 1380-1383.
- Hambley, J., Haywood, J., Rose, S.P.R. and Bateson, P.P.G. (1977). Effects of imprinting on lysine uptake and incorporation into protein in chick brain. *J. Neurobiol.* 8: 109-118.
- Hamori, J. (1973). The inductive role of presynaptic axons in the development of postsynaptic spines. *Brain Res.* 62: 337-344.
- Hanna, R.B., Hirano, A. and Pappas, G.D (1976). Membrane specialisations of dendritic spines and glia in the weaver mouse cerebellum. A freeze-fracture study. *J. Cell. Biol.* 68: 403-410.

- Hauser, K.F., McLaughlin, P.J. and Zagon, I.S. (1987). Endogenous opioids regulate dendritic growth and spine formation in the developing rat brain. *Brain Res.* 416: 157-161.
- Hebb, D.O. (1949). *The organization of behaviour*. Wiley, NY.
- Herndon, R.M. (1968). Thiophen induced granule cell necrosis in the rat cerebellum. An electron microscopic study. *Exp. Brain. Res.* 6: 49-57
- Herndon, R.M. Margolis, G. and Kilham, L. (1971). The synaptic organisation of malformed cerebellum induced by perinatal infection with the feline panleukopenia virus (PLV). *J. Neuropathol. Exp. Neurol.* 30: 557-570.
- Herschkowitz, M and Samuel, D. (1974). The influence of different behavioural situations on the incorporation of 5-³H-orotic acid into RNA in the newts, *Triturus cristatus* and *Pleurodeles waltl*. *Brain Res.* 75: 287-294.
- Herschkowitz, M. Wilson, J.E. and Glassman, E. (1975). The incorporation of radioactive amino acids into brain subcellular proteins during training. *J. Neurochem.* 25: 687-694.
- Hess, E.H. (1973). *Imprinting, early experience and the developmental psychobiology of attachment*. Van Nostrand Reinhold Co.: NY.
- Hinds, J.W. and Hinds, P.L. (1976). Synapse formation in the mouse olfactory bulb. I. Quantitative studies. *J. Comp. Neurol.* 169: 15-40.
- Hinkle, L., McCaig, D.D. and Robinson, K.R. (1981) The direction of growth of differentiating neurons and myoblasts from frog embryos in an applied electric field. *J. Physiol. (London)*. 314: 121-135.
- Hirano, A and Dembitzer, H.M. (1973). Cerebellar alterations in the weaver mouse. *J. Cell. Biol.* 56: 478-486.
- Hirano, A. and Dembitzer, H.M. (1974). Observations on the development of the weaver mouse cerebellum. *J. Neuropathol. Exp. neurol.* 33: 357-364.
- Hirano, A. and Dembitzer, H.M. (1975). Aberrant development of the Purkinje cell dendritic spine. In *Advances in Neuroethology*. 12. Ed: G.W. Kreutzberg. Raven: NY. pp 353-361.

- Hirano, A., Dembitzer, H.M. and Jones, M. (1972). An electron microscope study of cycasin-induced cerebellar alterations. *J. Neuropathol. Exp. Neurol.* 31: 113-125.
- Hirsch, H.V.B. and Spinnelli, D.N. (1971). Modification of the distribution of receptive field orientation in cats by selective visual exposure during development. *Exp. Brain Res.* 13: 509-527.
- Holloway, R.L. (1966). Dendritic branching: some preliminary results of training and complexity in rat visual cortex. *Brain Res.* 2: 393-396.
- Horn, G. (1985) *Memory, imprinting and the brain*. Clarendon press, Oxford.
- Horn, G., Bradley, P. and McCabe, B.J. (1985). Changes in the structure of synapses associated with learning. *J. Neurosci.* 5: 3161-3168.
- Horn, G., McCabe, B.J. and Cipello-Neto, J. (1983). Imprinting in the domestic chick: The role of each side of the hyperstriatum ventrale in acquisition and retention. *Exp. Brain Res.* 53: 91-98.
- Horn, G., Rose, S.P.R. and Bateson, P.P.G. (1973). Experience and plasticity in the nervous system. *Science.* 181: 506-514.
- Hornykiewicz, O. (1982). brain neurotransmitter changes in Parkinsons disease. *In Movement disorders*, Eds: C.D. Marsden and S. Fahn. Butterworth: London. pp 41-58.
- Horwitz, B. (1981). Neuronal plasticity: how changes in dendritic architecture can affect the spread of postsynaptic potentials. *Brain Res.* 224: 412-418.
- Howard, K.J., Rogers, L.J. and boura, A.L.A. (1980). Functional lateralisation of the chicken forebrain revealed by use of intracranial glutamate. *Brain Res.* 188: 369-382.
- Hubel, D.H., Wiesel, T.N. and LeVay, S. (1977). Plasticity of ocular dominance columns in monkey striate cortex. *Phil. Trans. R. Soc. London, Ser. B.* 278: 377-410.
- Hwang, H.M. and Greenough, W.T. (1984). Spine formation and synaptogenesis in rat visual cortex: a serial section developmental study. *Soc. for Neurosci. Abstr.* 10: 579.

- Hydén, H. and Egyhazi, E. (1962). Nuclear RNA changes during a learning experiment in rats. *Proc. Nat. Acad. Sci. USA*. 48: 1366-1373.
- Hydén, H. and Lange, P.W. (1970a). Brain cell protein synthesis specifically related to learning. *Proc. Nat. Acad. Sci. USA*. 65: 898-904.
- Hydén, H. and Lange, P.W. (1970b). S-100 protein; correlation with behaviour. *Proc. Nat. Acad. Sci. USA*. 67: 1959-1966.
- Hyman, B.T., van Hoesen, G.W. and Damasio, A.R. (1986). Glutamate depletion of the perforant pathway terminal zone in Alzheimers disease. *Soc. Neurosci. Abstr.* 12:994.
- Immelmann, K., Barlow, G.B., Main, M. and Petrinovich, L. (1979). (Eds.). *Behavioural development*. Cambridge University press: Cambridge, MA.
- Isaacson, R.L. and Pribram, K.H. (eds) (1975). *The Hippocampus. Vol.I and II*. Plenum: NY.
- Jack, J.J.B., Noble, D. and Tsien, R.W. (1975). *Electric current flow in excitable cells*. OUP, Clarendon: Oxford.
- Jaffe, L.F. and Poo, M.M (1979). Neurites grow faster towards the cathode than the anode in a steady field. *J. Exp. Zool.* 209: 115-128.
- Jellinger, K. (1986) Overview of morphological changes in Parkinsons disease. *Adv. Neurol.* 45: 1-18.
- Jones, W.H. and Thomas, D.B. (1962). Changes in the dendritic organisation of neurons in the cerebral cortex following deafferentation. *J. Anat.* 96: 375-381.
- Juraska, J.M. and Fifkova, E. (1979a). A Golgi study of the early postnatal development of the visual cortex of the hooded rat. *J. Comp. Neurol.* 183: 247-256.
- Juraska, J.M. and Fifkova, E. (1979b). A electron microscope study of the early postnatal development of the visual cortex of the hooded rat. *J. Comp. Neurol.* 183: 257-267.
- Kaczmarek, L.K., Strong, J.A. and Kauer, J.A. (1986). The role of protein

- kinases in the control of prolonged changes in neuronal excitability. *Prog. Brain Res.* 69: 77-90.
- Kandel, E.R. and Schwartz, J.H. (1982). Molecular biology of learning : modulation of neurotransmitter release. *Science* 218: 433-443.
- Katsumaru, H., Murakami, F. and Tsukahara, N. (1982). Actin filaments in dendritic spines of red nucleus neurons demonstrated by immunoferritin localisation and heavy meromyosin binding. *Biomed. Res.* 3:337-340.
- Kelso, S.R., Ganong, A.H. and Brown, T.H. (1986). Hebbian synapses in hippocampus. *Proc. Natl. Acad. Sci. USA.* 83: 5326-5330.
- Kiss, J. (1977). Synthesis and transport of newly synthesised proteins in dendrites of rat hippocampal pyramidal cells. An electron microscope and autoradiographic study. *Brain Res.* 124: 237-250.
- Koch, C. and Poggio, T. (1983a). Electric properties of dendritic spines. *TINS* 6:80-83.
- Koch, C. and Poggio, T. (1983b). A theoretical analysis of the electrical properties of dendritic spines. *Proc R. Soc. Lond. Ser. B.* 218: 455-477.
- Kostyuk, P.G. (1986). Cyclic nucleotides as modulators and activators of ionic channels in the nerve cell membrane. *Prog. Brain Res.* 69: 133-140.
- Krivanek, J. (1974). Are the cortical proteins involved in visual discrimination training? *Ergeb. Exp. Med.* 17: 595-611.
- Krivanek, J. (1975). Labelling of TCA soluble fraction and protein of the rat visual cortex after learning a pattern discrimination task. *Experientia.* 31: 1042-1043.
- Kuhnt, U., Mihaly, A. and Joo, R. (1985). Increased binding of calcium in the hippocampus during long-term potentiation. *Neurosci. Letts.* 53: 149-154.
- Kupferman, I. (1980). The role of cyclic nucleotides in excitable cells. *Ann. Rev. Physiol.* 42: 629-641.
- Landis, D.M.D. and Reese, T.S. (1983). Cytoplasmic organisation in cerebellar dendritic spines. *J. Cell. Biol.* 97: 1169-1178.

- Larramendi, L.M.H. (1969). Analysis of synaptogenesis in the cerebellum of the mouse. In *Neurobiology of cerebellar evolution and development*, Ed: R. Llinas. American Medical Association.
- Lebeaux, Y.J. and Willemot, J. (1975). An ultrastructural study of the microfilaments in rat brain by means of EPTA staining and heavy meromyosin labelling. II. The synapses. *Cell and Tissue Res.* 160: 37-68.
- Lee, K.S., Oliver, M., Schottler, F. and Lynch, G. (1981). Electron microscope studies of brain slices: The effects of high frequency stimulation on dendritic ultrastructure. In *Electrical activity in isolated mammalian CNA preparations*, Eds: G.A. Kerkut and H.B. Wheal. Academia: NY. pp 189-212.
- Lee, K.S., Schottler, F., Oliver, M. and Lynch, G. (1980). Brief bursts of high frequency stimulation produces two types of structural change in the rat hippocampus. *J. neurophysiol.* 44: 247-258.
- Lemos, J.R., Novak-Hofer, I. and Levitan, I.B. (1986). Regulation of ion channel activity by protein phosphorylation. *Prog. Brain Res.* 69: 107-118.
- LeVay, S. (1973). Synaptic patterns in the visual cortex of the cat and monkey: electron microscopy of Golgi preparations. *J. Comp. Neurol.* 150: 53-86.
- Llinas, R. (1975). The cortex of the cerebellum. *Sci. Am. Jan 1975*: 56-71.
- Llinas, R and Hillman, D.E. (1969). Physiological and morphological organisation of the cerebellar circuits in various vertebrates. In *Neurobiology of cerebellar evolution and development*. ED; R. Llinas. American Medical Association, USA.
- Llinas, R., Hillman, D.E. and Precht, W. (1973). Neuronal circuit organisation in mammalian agranular cortex. *J. neurobiol.* 4: 69-94.
- Lund, J.S. and Lund, R.D. (1970). The termination of callosal fibres in the paraviscual cortex of the rat. *Brain Res.* 17: 25-45.
- Lund, J.S., Boothe, R.G. and Lund, R.D. (1977). Development of neurons in the visual cortex of the monkey (*macaca nemestrina*). A Golgi study from foetal day 127 to postnatal maturity. *J. Comp. Neurol.* 176: 149-188.
- Lynch, G. (1986). *Synapses, circuits and the beginnings of memory*. MIT:

Cambridge, Mass. pp 122-.

- Lynch, G. and Baudry, M. (1984). The biochemistry of learning and memory: a new and specific hypothesis. *Science*. 224: 1057-1063.
- Mahajan, D.S. and Desiraju, T. (1988). Alterations of dendritic branching and spine densities of hippocampal CA3 pyramidal neurons induced by operant conditioning in the phase of the brain growth spurt. *Exp. Neurol.* 100(1): 1-15.
- Marin-Padilla, M. (1972). Structural abnormalities of the cerebral cortex in human chromosomal aberrations: a Golgi study. *Brain Res.* 44: 625-629.
- Marin-Padilla, M. (1974). Structural organisation of the cerebral cortex (motor cortex) in human chromosomal aberrations: a Golgi study. *Brain Res.* 66: 375-391.
- Marsh, G. and Beams, H.W. (1946). In vitro control of growing chick nerve fibres by applied electric currents. *J. Cell. Comp. Physiol.* 27: 139-157.
- Mason, R.J., and Rose, S.P.R. (1987). Lasting changes in spontaneous multi-unit activity in the chick brain following passive avoidance training. *Neurosci.* 21(3): 931-941.
- Mason R.J. and Rose, S.P.R. (1988). passive avoidance learning produces focal elevation of bursting activity in the chick brain: amnesia abolishes the increase. *Beh. and Neural Biol.* 49: 280-292.
- Mates, S.L. and Lund, J.S. (1983a). Neuronal composition and development in lamina 4c of monkey striate cortex. *J. Comp. Neurol.* 221: 60-90.
- Mates, S.L. and Lund, J.S. (1983b). Developmental changes in the relationship between type 1 synapses and spiny neurons in the mokey visual cortex. *J. Comp. Neurol.* 221: 91-97.
- Mates, S.L. and Lund, J.S. (1983c). Developmental changes in the relationship between type 2 synapses and spiny neurons in the mokey visual cortex. *J. Comp. Neurol.* 221: 98-105.
- Mattson, M.P. (1988). Neurotransmitters in the regulation of neuronal cytoarchitecture. *Brain Res. Rev.* 13: 179-212.

- Mattson, M.P. and Kater, S.B. (1987). Calcium regulation of neurite elongation and growth cone motility. *J. Neurosci.* 7: 4034-4043.
- Mattson, M.P., Dou, P. and Kater, S.B. (1987). Pruning of hippocampal pyramidal neuron dendritic architecture in vitro by glutamate and a protective effect of GABA plus diazepam. *Soc. neurosci. Abstr.* 13: 367.
- Matus, A. (1988). Microtubule-associated proteins: their potential role in determining neuronal morphology. *Ann. Rev. Neurosci.* 11: 29-44.
- McCabe, B.J. and Horn, G. (1988). Learning and memory: regional changes in N-methyl-D-aspartate receptors in the chick brain after imprinting. *Proc. Nat. Acad. Sci. USA.* in press.
- McCabe, N.R. and Rose, S.P.R. (1984). Passive avoidance training increases fucose incorporation into glycoproteins in chick forebrain slices *in vitro*. *Neurochem. Res.* 10(8): 1083-1095.
- McConnell, P. and Berry, M. (1978a). Effects of undernutrition on Purkinje cell dendritic growth in the rat. *J. Comp. Neurol.* 177: 159-172.
- McConnell, P. and Berry, M. (1978b). The effects of refeeding after neonatal starvation on Purkinje cell dendritic growth in the rat. *J. Comp. Neurol.* 178: 759-772.
- McNeill, T.H., Brown, S.A., Rafols, J.A. and Shoulson, I. (1988). Atrophy of medium spiny I striatal dendrites in advanced Parkinsons disease. *Brain Res.* 455: 148-152.
- Mehraein, P., Yamada, M., Tarnowska, D and Ziduszco E. (1975). Quantitative study of dendrites and dendritic spines in Alzheimers disease and senile dementia. In *Physiology and pathology of dendrites*, Ed: G.W. Kreutzberg. Raven press: NY. (Adv. neurol. 12: 453-458).
- Mench, J.A. and Andrew, R.J. (1986). Lateralisation of a food search task in the domestic chick. *Beh. and Neural Biol.* 46: 115-122.
- Mikeladze, A.L. (1969). Characterisation of the postsynaptic structures of the brain. *Folia Morphologica* 17: 420-431.

- Mileusnic, R., Rose, S.P.R. and Tillson, P. (1981). Passive avoidance training results in changes in concentration of and incorporation into colchicine binding proteins in the chick forebrain. *J. Neurochem.* 34: 1007-1015.
- Miller, M. and Peters, A. (1981). Maturation of rat visual cortex. II. A combined Golgi electron microscope study of pyramidal neurons. *J. Comp. Neurol.* 203: 555-573.
- Miller, J.P., Rall, W. and Rinzel, J. (1985). Synaptic amplification by active membrane in dendritic spines. *Brain Res.* 325: 325-330.
- Minkwitz, H-G. (1976). Zur Entwicklung der neuronenstruktur des hippocampus wahrend der pra - und postnatalen ontogenese der albinoratte. III Mitteilung: morphometrische erfassung der ontogenetischen veränderungen in dendritenstruktur und spine besatz an pyramidenneuronen (CA1) des hippocampus. *J. Hirnforschung.* 17:255-275.
- Moore, B.W. (1972). Chemistry and biology of two proteins S-100 and 14-3-2, specific to the nervous system. *Int. Rev. Neurobiol.* 15: 215-225.
- Morest, D.K. (1969). The growth of dendrites in the mammalian brain. *Z. Anat. Entwicklungsgesch.* 138: 290-317.
- Muller, G.E. and Pilzecker, A. (1900). Experimentelle beiträge zur lehre vom gedächtnis. *Z. Psychol. Suppl.* 1:1-288. (Germany).
- Nottebohm, F. (1977). Asymmetries in neural control of vocalization in the canary. In *Lateralization in the nervous system* (eds. S.Harnard, R.W.Doty, L. Goldstein, J. Jaynes and G. Krauthamer) Academic press. pp23-45.
- Nottebohm, F. and Arnold, A.P. (1976). Sexual dimorphism in vocal control areas of the songbird brain. *Science.* 194: 211-213.
- Nunzi, M.G., Milan, F., Guidolin, D. and Toffano, G. (1987). Dendritic spine loss in hippocampus of aged rats. Effects of brain phosphatidylserine administration. *Neurobiology of Aging.* 8: 501-510.
- Palay, S.L. and Chan-Palay, V. (1974). *Cerebellar cortex: cytology and organisation.* Springer-Verlag; Berlin-Heidelberg.
- Parnavelas, J.G. and Globus, A. (1976). The effect of continuous illumination on the development of cortical neurons in the rat: A Golgi study. *Exp. Neurol.*

51: 637-647.

Parnavelas, J.G., Globus, A. and Kaups, P. (1973). Continuous illumination from birth affects spine density of neurons in the visual cortex of the rat. *Exp. Neurol.* 40: 742-747.

Parnavelas, J.G., Lynch, G., Brecha, N., Cotman, C.W. and Globus, A. (1974). Spine loss and regrowth in hippocampus following deafferentation. *Nature.* 248: 71-73.

Pastenak, J.F. and Woolsey, T.A. (1975). On the "selectivity" of the Golgi-Cox method. *J. Comp. Neurol.* 160: 307-312.

Patel, N.B. and Poo, M.M. (1982). Orientation of neurite growth by extracellular electric fields. *J. Neurosci.* 2: 483-496.

Patterson, T.A., Alvarado, M.C., Warner, I.T., Bennett, E.L. and Rosenzweig, M.R. (1986). Memory stages and brain asymmetry in chick learning. *Beh. Neurosci.* 100(6): 856-865.

Pedata, F., Giovanelli, L., Spignoli, G., Giovannini, G. and Pepev, G. (1985). Phosphatidylserine increases acetylcholine release from cortical slices in aged rats. *Neurobiol. Aging.* 6: 337-339.

Perkel, D.H. and Perkel, D.J. Dendritic spines: role of active membrane in modulating synaptic efficacy. *Brain Res.* 325 (1985) 331-335.

Peters, A. and Feldman, M.L. (1976). The projection of the lateral geniculate nucleus to area 17 of the rat cerebral cortex. I. General description. *J. Neurocytol.* 5:63-84.

Peters, A. and Kaiserman-Abramof, I.R. (1970). The small pyramidal neuron of the rat cerebral cortex. the perikaryon, dendrites and spines. *Am. J. Anat.* 127: 321-356.

Phillips, R.E. and Youngren, O.M. (1986). Unilateral kainic acid lesions reveal dominance of right archistriatum in avian fear behaviour. *Brain Res.* 377: 216-220.

Pinsker, H.M., Hening, W.A., Carew, T.J. and Kandel, E.R. (1973). Long-term sensitization of a defensive withdrawal reflex in *Aplysia*. *Science (Wash.*

D.C.) 182: 1039-1042.

Pinsker, H.M., Kupferman, F., Castelluci, U.F. and Kandel, E.R. (1970). Habituation and dishabituation of the gill withdrawal reflex in *Aplysia*. *Science*. 167: 1740-1742.

Pongracz, F. The function of dendritic spines: A theoretical study. *Neurosci.* 15 (no. 4) (1985) 933-946.

Privat, A. (1975). Dendritic growth *in vitro*. *Adv. Neurol.* 12: 201-216.

Privat, A and Drian, M.J. (1974). Première analyse ultrastructurale de la plasticité des circuits cérébelleux *in vitro*. *C.R. Acad. Sci. (Paris)* 278: 659-662.

Purpura, D.P. (1974). Dendritic spine "dysgenesis" and mental retardation. *Science*. 186: 1126-1128.

Purves, D. and Hadley, R.D. (1985). Changes in the dendritic branching of adult mammalian neurons revealed by repeated imaging *in situ*. *Nature*. 315: 404-406.

Quinn, W.G., Sziber, P.P. and Booker, R. (1979). The drosophila memory mutant *amnesiac*. *Nature*. 227: 212-214.

Rakic, P. (1974). Intrinsic and extrinsic factors influencing the shape of neurons and their assembly into neuronal circuits. In *Frontiers in neurology and neuroscience research*, Eds: P.Seeman and G.M. Brown. University of Toronto press. pp 112-132.

Rakic, P and Sidman, R.L. (1973). organization of cerebellar cortex secondary to deficit of granule cells in weaver mutant mice. *J. Comp. neurol.* 152: 133-162.

Rall, W. and Segev. I. (1987). Functional possibilities for synapses on dendrites and on dendritic spines. In *Synaptic function*. Eds: G.M. Edelman, W.E. Gall and W.M. Cowan. NY: Wiley. 605-636.

Ramon y Cajal, S. (1911). In *Histologie du système nerveux de l'homme et de vertébrés*, vol. 2. pp 886-90. Maloine, Paris. [republished 1955, *Histologie du système nerveux*. Instituto Ramon y Cajal, Madrid].

Rasmussen, H. (1981). *Calcium and cyclic AMP as synarchic messengers*. Wiley:

NY.

- Rausch, G. and Scheich, H. (1982). Dendritic spine loss and enlargement during maturation of the speech control system in the mynah bird (*Gracula religiosa*). *Neurosci. Letts.* 29: 129-133.
- Rees, R.P., Bunge, M.B. and Bunge, R.P. (1976). Morphological changes in the neuritic growth cone and target neuron during synaptic junction development in culture. *J. Cell Biol.* 68: 240-263.
- Rehn, B., Breipohl, W., Mendoza, A.S. and Apfelbach, R. (1986). Changes in granule cells of the ferret olfactory bulb associated with imprinting on prey odours. *Brain Res.* 373: 114-125.
- Rehn, B., Panhuber, H., Laing, D.G. and Breipohl, W. (1988). Spine density on olfactory granule cell dendrites is reduced in rats reared in restricted olfactory environments. *Dev. Brain Res.* 40: 143-147.
- Robinson, H. and Koch, C. (1984). An information storage mechanism: calcium and spines. *Artificial Intelligence Memo.* 779 (CBID paper 004), pp1-14. Cambridge, MA: MIT press.
- Rogers, L.J. and Anson, J.M. (1979). Lateralisation of function in the chicken forebrain. *Pharmac. Biochem. Behav.* 10(5): 679-686.
- Rogers, L.J. and Ehrlich, D. (1983). Asymmetry in the chicken forebrain during development and a possible involvement of the supraoptic decussation. *Neurosci. Letts.* 37: 123-127.
- Rogers, L.J. and Sink, H.S. (1984). Transient asymmetry in visual pathways of young chickens. *Neurosci. Letts.* 27: S116.
- Rose, S.P.R. and Csillag, A. (1985). passive avoidance training results in lasting changes in deoxyglucose metabolism in the left hemisphere. *Beh and Neural Biol.* 44(2): 315-324.
- Rose, S.P.R. and Harding, S. (1984). Training increases ³H-fucose incorporation in chick brain only if followed by memory storage. *Neurosci.* 12: 663-667.
- Rose, S.P.R. and Haywood, J. (1976). Experience, learning and brain metabolism. In *Biochemical correlates of brain structure and function*. Ed:

- Davison, A.N. pp. 249-292. Academic press: London.
- Rose, S.P. R., Gibbs, M.E. and Hambley, J.W. (1980). Transient increase in forebrain muscarinic cholinergic receptors following passive avoidance learning in the young chick. *Neurosci.* 5: 169-172.
- Rothblat, L.A. and Schwartz, M. (1979). The effect of monocular deprivation on dendritic spines in visual cortex of young and adult albino rats: evidence for a sensitive period. *Brain Res.* 161:156-161.
- Routtenberg, A. (1986). Synaptic plasticity and protein kinase C. *Prog. Brain Res.* 69: 211-234.
- Ruiz-Marcos, A. and Valverde, F. (1969). The temporal evolution of the distribution of dendritic spines in visual cortex of normal and dark-reared mice. *Exp. Brain Res.* 8: 284-294.
- Sadler, M. and Berry, M. (1983). Morphometric study of the development of Purkinje cell dendritic trees in the mouse using vertex analysis. *J. Microscopy* 131(3): 341-354.
- Sattilaro, R.F., Dentler, W.L. and LeCluyse, E.L. (1981). Microtubule associated proteins (MAPs) and the organisation of actin filaments in vitro. *J. Cell Biol.* 90: 467-473.
- Sattilaro, W. (1986). Interaction of microtubule associated protein 2 with actin filaments. *Biochemistry.* 25: 2003-9.
- Scheibel, M.E. and Scheibel, A.B. (1968). On the nature of dendritic spines - report of a workshop. *Comm. in Beh. Biol. Part A, 1*: 231-265.
- Schubert, D., Lacorbiere, M., Whitlock, C. and Stallay, W. (1978). Alterations in the surface properties of cells responsive to nerve growth factor. *Nature (London).* 273: 718-723.
- Scott, J.P. (1962). Critical periods in behavioural development. *Science*, 138: 949-957.
- Seil, F.G. (1979). Cerebellum in tissue culture. *Rev of Neurosci.* 4: 105-178.
- Shapiro, S. and Vukovich, K.R. (1970). Early experience effects upon cortical dendrites: A proposed model for development. *Science.* 167: 292-294.

- Shashoua, V.E. (1982). Molecular and cell biological aspects of learning: Toward a theory of memory. *Adv. Cell. Neurobiol.* 3: 97-141.
- Shepherd, G.M. and Brayton, R.K. (1987). Logic operations are properties of computer simulated interactions between excitable dendritic spines. *Neurosci.* 21 no. 1 (1987) 151-165.
- Shepherd, G.M., Brayton, R.K., Miller, J.P., Segev, I., Rinzel, J. and Rall, W. (1985). Signal enhancement in distal cortical dendrites by means of interactions between active dendritic spines. *Proc. Natl. Acad. Sci. USA.* 82: 2192-2195.
- Sholl, D.A. (1956). *Organization of the cerebral cortex*. Methuen: London.
- Shüz, A. (1978). Some facts and hypotheses concerning dendritic spines and learning. In *Architectonics of the cerebral cortex*, Eds: M.A.B. Brazier and H. Petsche. Raven press: NY.
- Sirevaag, A.M. and Greenough, W.T. (1985). Differential rearing effects on rat visual cortex synapses. II. Synaptic morphometry. *Brain Res.* 351(2): 215-226.
- Sotelo, C. (1973). Permanence and fate of paramembranous synaptic specialisations in "mutant" and experimental animals. *Brain Res.* 62: 345-351.
- Sotelo, C. (1975). Anatomical, physiological and biochemical studies of the cerebellum from mutant mice. II. Morphological study of cerebellar cortical neurons and circuits in the weaver mouse. *Brain Res.* 94: 19-44.
- Sotelo, C. (1978). Purkinje cell ontogeny: formation and maintenance of spines. *Prog. Brain. Res.* 48: 149-170.
- Sotelo, C., Hillman, D.E., Zamora, A.K. and Llinas, R. (1975). Climbing fibre deafferentation: its action on Purkinje cell dendritic spines. *Brain Res.* 98: 574-581.
- Steward, O. (1983). Alterations in polyribosomes associated with dendritic spines during the reinnervation of the dentate gyrus of the adult rat. *J. Neurosci.* 3(1): 177-188.

- Steward, O. and Fass, b. (1983). Polyribosomes associated with dendritic spines in the denervated dentate gyrus: evidence for local regulation of protein synthesis during re-innervation. *Prog. Brain Res.* 58: 131-136.
- Steward, O. and Vinsant, S.L. (1983). The process of reinnervation in the dentate gyrus of the adult rat: a qualitative electron microscopic analysis of terminal proliferation and reactive synaptogenesis. *J. Comp. Neurol.* 214: 370-386.
- Stewart, M.G., Rose, S.P.R. and King, T.S. (1984). A stereological investigation of synapses in chick paleostriatum augmentatum and lobus parolfactorious following training on a passive avoidance task. *Acta. Stereologica.* 2(Suppl. 1): 227-230.
- Stirling, R.V. and Bliss, T.V.P. (1978). Observations on the commissural projection to the dentate gyrus in the reeler mutant mouse. *Brain Res.* 150: 447-465.
- Sukumar, R., Rose, S.P.R. and Burgoyne, R.D. (1980). Increased incorporation of fucose into chick brain glycoprotein following training on a passive avoidance task. *J. Neurochem.* 34: 1000-1006.
- Swindale, N.V. (1981). Dendritic spines only connect. *TINS* 4:240-241.
- Szentagothai, J. and Hamori, J. (1969). Growth and differentiation of synaptic structures under circumstances of deprivation of function and of lack in distant connections. In *Cellular dynamics of the neuron*, Ed: S.H.Barondes. Academic press: NY. pp 301-320.
- Tanzi, E. (1893). I fatti e le induzioni nell' odierna istologia del sistema nervoso. *Riv. Sper. Freniat. Med. Leg. Alien. Ment.* 19: 419-72.
- Toffano, G., Leon, A., mazzari, S., Savoini, G., Teolato, S. and Orlando, P. (1978). Modification of noradrenergic hypothalamic system in rat injected with phosphatidylserine liposomes. *Life Sci.* 23: 1093-1102.
- Tombol, T., Csillag, A. and Stewart, M.G. (1987). Cell types of the hyperstriatum ventrale of the domestic chicken, *Gallus domesticus*: a Golgi study. *J. Hirnforschung*.
- Turner, B. (1968). Pathology of paralysis agitans. In *Diseases of the basal ganglia*, Eds:P.J. Vinken and G.W. Bruyn. Northe Holland; Amsterdam. pp

212-217.

- Turner, A.M. and Greenough, W.T. (1985). Synapses per neuron and synaptic dimensions in occipital cortex of rats reared in complex, social or isolated housing. *Acta. Steoreologica Supplementum. 2*: 239-244.
- Valverde, F. (1967). Apical dendritic spines of the visual cortex and light deprivation in the mouse. *Exp. Brain Res. 3*: 337-352.
- Valverde, F. (1970). The Golgi method: A tool for comparative structural analyses. *In Contemporary research methods in neuroanatomy*, Eds: W.J.H. Nauta and S.O.E. Ebbesson. Springer-Verlag: London and NY.
- Valverde, F. (1971). Rate and extent of recovery from dark rearing in the mouse. *Brain. Res. 33*: 1-11.
- Van Harreveld, A. and Fifkova, E. (1975). Swelling of dendritic spines in the fascia dentata after stimulation of the perforant fibres as a machanism of post-tetanic potentiation. *Exp. Neurol. 49*: 736-749.
- Vaughn, J.E., Hendrickson, C.K. and Grieshaber, J.A. (1974). A quantitative study of synapses or motor neuron dendritic growth cones in developing mouse spinal cord. *J. Cell. Biol. 60*: 664-672.
- Volkmar, F.R. and Greenough, W.T. (1972). Rearing complexity affects branching of dendrites in the visual cortex of the rat. *Science. 176*: 1445-1447.
- von Noorden, G.K. (1973). Experimental amblyopia in monkeys. Further behavioural observations and clinical correlations. *Invest. Ophthal., 12*: 721-726.
- von Noorden, G.K., Dowling, J.E. and Ferguson, D.C. (1970). Experimental amblyopia in monkeys. I. Behavioural studies of stimulus deprivation amblyopia. *Arch. Ophthal. (Chicago). 84*: 206-214.
- Walker, S.F. (1980). Lateralization of function in the vertebrate brain: a review. *The British Journal of Psychology 71*: 329-369.
- Wallhauser, E and Scheich, H. (1987). Auditory imprinting leads to differential 2-deoxyglucose uptake and dendritic spine loss in the chick rostral forebrain. *Dev. Brain. Res. 31*: 29-44.

- Wenzel, J., Kammerer, E., Joschko, R., Joschko, M., Kaufman, W., Kirsche, W., and Matthies, H. (1977). The influence of a learning experience on synaptosomes in the rat hippocampus. *Z. N. Am. Forsch. Leipzig*. 91: 57-73.
- Wilson, C.J., Groves, P.M., Kitai, S.T. and Linder, J.C. (1983). Three dimensional structure of dendritic spines in the rat neostriatum. *J. Neurosci.* 3: 383-398.
- Wilson, C.J. (1984). Passive cable properties of dendritic spines and spiny neurons. *J. Neurosci.* 4: 281-297.
- Winfield, D.A. (1981). The postnatal development of synapses in the visual cortex of the cat and the effects of eyelid closure. *Brain Res.* 206: 166-171.
- Yamamoto, H., Fukunaga, K., Goto, S., Tanaka, E. and Miyamoto, E. (1985). Calcium, calmodulin-dependent regulation of microtubule formation via phosphorylation of microtubule associated protein 2, τ factor and tubulin, and comparison with the cyclic AMP-dependent phosphorylation. *J. Neurochem* 44: 759-768.
- Zanotti, A., Aporti, F., Rubini, R. and Toffano, G. (1986). Learning and memory deficits in rats during aging: effect of phosphatidylserine treatment. In *Modulation of central and peripheral transmitter function*, Eds: G. Biggio, P.F. Spano, G. Toffano and G. L. Gessa. Liviana press, Padova. pp 320-323.
- Zimmer, J. (1978). Development of the hippocampus and the fascia dentata. Morphological and histochemical aspects. *Prog. Brain Res.* 48: 171-190.

APPENDIX A

TABLES

The data for the experiments are presented in a tabulated form for easy reference. The legends for tables 7 to 10 are given with the tables. Here is a summary of the legends for tables 1 to 6, which refer to the mean values (in μm) for the first spine density experiment (chapter 3), and a summary of the results of the ANOVA test. The means are presented with \pm the standard errors of those means which were averaged over data from 8 control and 9 trained chicks in this experiment. EXP refers to the effects of training and HEM, the effects of hemisphere.

TABLE 1: Spine density (SD),

TABLE 2: Dendrite diameter (DD),

TABLE 3: Dendrite length (DL),

TABLE 4: 'Overall spine length' (SL),

TABLE 5: Spine head diameter (SHD),

TABLE 6: Spine stem length (STL).

TABLE 7: Data from the amnesia experiment discussed in chapter 4,

TABLE 8: Dendritic branching summary data on *changes in the number of branches* after training on LMPNs in the IMHV.

TABLE 9: Dendritic branching summary data on *changes in the number of ring intersections* after training on LMPNs.

TABLE 10: Spearman correlations between visible spine density, spine length and dendrite diameter.

TABLE 11: Data from the topological, vertex, analysis.

TABLE 1

SPINE DENSITY DATA

HEM	ORDER	TRAINED	CONTROL
LEFT	2	2.1591 \pm 0.273	2.0704 \pm 0.206
	3	3.0178 \pm 0.788	2.5825 \pm 0.289
	4	2.4375 \pm 1.183	1.8810 \pm 0.555
	5	1.1743 \pm 0.983	0.8721 \pm 0.376
	6	0.3030 \pm 0.468	0.1789 \pm 0.164
	7	0.0279 \pm 0.079	0.0416 \pm 0.118
RIGHT	2	1.9650 \pm 0.108	2.0423 \pm 0.182
	3	2.5816 \pm 0.696	2.4753 \pm 0.656
	4	1.8090 \pm 1.095	1.8911 \pm 0.843
	5	1.0592 \pm 0.648	0.7182 \pm 0.640
	6	0.2244 \pm 0.213	0.1320 \pm 0.172
	7	0.0350 \pm 0.065	0.0166 \pm 0.047

ANOVA	F-VALUE	SIG.
EXPERIMENTAL EFFECTS (EXP):	F(1, 15) = 15407	p < 0.0001
HEMISPHERE EFFECTS (HEM):	F(1, 15) = 38.5	p < 0.0001
EXP BY HEM:	F(1, 15) = 17.85	p < 0.001
ORDER:	F(3, 45) = 4.827	p < 0.005
EXP BY ORDER:	F(3, 45) = 0.172	n.s.
ORDER WITHIN HEM	F(6, 90) = 836.5	p < 0.0001
EXP BY ORDER WITHIN HEM	F(6, 90) = 50.93	p < 0.0001

TABLE 2

DENDRITE DIAMETER DATA

HEM	ORDER	TRAINED	CONTROL
LEFT	2	1.1746 \pm 0.039	1.1329 \pm 0.086
	3	1.0817 \pm 0.042	1.1425 \pm 0.064
	4	0.9727 \pm 0.027	0.9936 \pm 0.066
	5	0.8770 \pm 0.046	1.0018 \pm 0.062
	6	0.6929 \pm 0.032	0.7563 \pm 0.021
RIGHT	2	1.1942 \pm 0.063	1.2538 \pm 0.061
	3	0.9742 \pm 0.040	1.0695 \pm 0.025
	4	0.8708 \pm 0.053	0.9145 \pm 0.024
	5	0.7548 \pm 0.066	0.8350 \pm 0.026
	6	0.7167 \pm 0.044	0.7099 \pm 0.037

ANOVA	F-VALUE	SIG.
EXPERIMENTAL EFFECTS (EXP):	F(1, 15) = 1.91	n.s.
HEMISPHERE EFFECTS (HEM):	F(1, 15) = 4.07	p < 0.07
EXP BY HEM:	F(1, 15) = 0.20	n.s.
ORDER:	F(3, 45) = 35.54	p < 0.0001
EXP BY ORDER:	F(3, 45) = 0.79	n.s.
ORDER WITHIN HEM	F(6, 90) = 835.22	p < 0.0001
EXP BY ORDER WITHIN HEM	F(6, 90) = 1.01	n.s.

TABLE 3

DENDRITE LENGTH DATA

HEM	ORDER	TRAINED	CONTROL
LEFT	2	29.956 \pm 3.204	37.875 \pm 3.951
	3	37.633 \pm 3.832	41.100 \pm 2.876
	4	36.611 \pm 1.774	37.000 \pm 3.220
	5	32.278 \pm 2.074	36.550 \pm 3.058
	6	32.800 \pm 2.206	40.030 \pm 3.802
RIGHT	2	26.444 \pm 2.548	26.138 \pm 2.266
	3	35.589 \pm 2.037	30.100 \pm 1.778
	4	37.589 \pm 3.815	37.150 \pm 1.734
	5	29.567 \pm 3.913	33.850 \pm 1.869
	6	38.180 \pm 4.690	36.040 \pm 2.400

ANOVA	F-VALUE	SIG.
EXPERIMENTAL EFFECTS (EXP):	F(1, 15) = 0.77	n.s.
HEMISPHERE EFFECTS (HEM):	F(1, 15) = 11.27	p < 0.005
EXP BY HEM:	F(1, 15) = 3.44	n.s.
ORDER:	F(3, 45) = 6.76	p < 0.002
EXP BY ORDER:	F(3, 45) = 1.20	n.s.
ORDER WITHIN HEM	F(6, 90) = 327.91	p < 0.0001
EXP BY ORDER WITHIN HEM	F(6, 90) = 0.87	n.s.

TABLE 4

'OVERALL' SPINE LENGTH DATA

HEM	ORDER	TRAINED	CONTROL
LEFT	2	1.3926 \pm 0.036	1.4435 \pm 0.027
	3	1.3652 \pm 0.027	1.4346 \pm 0.039
	4	1.3540 \pm 0.030	1.4128 \pm 0.020
	5	1.3346 \pm 0.021	1.2914 \pm 0.041
	6	1.2336 \pm 0.027	1.3678 \pm 0.072
RIGHT	2	1.3173 \pm 0.027	1.4007 \pm 0.050
	3	1.3517 \pm 0.025	1.3979 \pm 0.029
	4	1.4046 \pm 0.033	1.3830 \pm 0.025
	5	1.3960 \pm 0.039	1.3847 \pm 0.027
	6	1.3283 \pm 0.037	1.3478 \pm 0.052

ANOVA	F-VALUE	SIG.
EXPERIMENTAL EFFECTS (EXP):	F(1, 15) = 1.92	n.s.
HEMISPHERE EFFECTS (HEM):	F(1, 15) = 0.00	n.s.
EXP BY HEM:	F(1, 15) = 0.04	n.s.
ORDER:	F(3, 45) = 1.99	n.s.
EXP BY ORDER:	F(3, 45) = 2.76	p < 0.06
ORDER WITHIN HEM	F(6, 90) = 4251.93	p < 0.0001
EXP BY ORDER WITHIN HEM	F(6, 90) = 1.41	n.s.

TABLE 5

SPINE HEAD DIAMETER DATA

HEM	ORDER	TRAINED	CONTROL
LEFT	2	0.7259 \pm 0.013	0.6894 \pm 0.010
	3	0.7361 \pm 0.014	0.6785 \pm 0.016
	4	0.7260 \pm 0.012	0.6965 \pm 0.013
	5	0.7237 \pm 0.009	0.6651 \pm 0.008
	6	0.6847 \pm 0.008	0.6783 \pm 0.029
RIGHT	2	0.7078 \pm 0.014	0.6950 \pm 0.009
	3	0.7154 \pm 0.013	0.7067 \pm 0.008
	4	0.7244 \pm 0.009	0.7000 \pm 0.010
	5	0.7146 \pm 0.010	0.6945 \pm 0.013
	6	0.6900 \pm 0.030	0.6736 \pm 0.016

ANOVA	F-VALUE	SIG.
EXPERIMENTAL EFFECTS (EXP):	F(1, 15) = 10.52	p < 0.006
HEMISPHERE EFFECTS (HEM):	F(1, 15) = 0.19	n.s
EXP BY HEM:	F(1, 15) = 8.74	p < 0.02
ORDER:	F(3, 45) = 1.04	n.s
EXP BY ORDER:	F(3, 45) = 0.39	n.s
ORDER WITHIN HEM	F(6, 90) = 9272.17	p < 0.0001
EXP BY ORDER WITHIN HEM	F(6, 90) = 5.89	p < 0.0001

TABLE 6

SPINE STEM LENGTH DATA.

HEM	ORDER	TRAINED	CONTROL
LEFT	2	0.6667 \pm 0.041	0.7541 \pm 0.023
	3	0.6291 \pm 0.021	0.7559 \pm 0.031
	4	0.6280 \pm 0.030	0.7162 \pm 0.019
	5	0.6109 \pm 0.028	0.6263 \pm 0.035
	6	0.5489 \pm 0.030	0.6895 \pm 0.058
RIGHT	2	0.6096 \pm 0.020	0.7057 \pm 0.048
	3	0.6362 \pm 0.017	0.6911 \pm 0.030
	4	0.6852 \pm 0.026	0.6830 \pm 0.027
	5	0.6814 \pm 0.035	0.6903 \pm 0.026
	6	0.6383 \pm 0.021	0.6716 \pm 0.041

ANOVA	F-VALUE	SIG.
EXPERIMENTAL EFFECTS (EXP):	F(1, 15) = 11.69	p < 0.005
HEMISPHERE EFFECTS (HEM):	F(1, 15) = 0.00	n.s
EXP BY HEM:	F(1, 15) = 0.83	n.s
ORDER:	F(3, 45) = 1.07	n.s
EXP BY ORDER:	F(3, 45) = 2.02	n.s
ORDER WITHIN HEM	F(6, 90) = 1196.41	p < 0.0001
EXP BY ORDER WITHIN HEM	F(6, 90) = 3.41	p < 0.005

PARAMETER	HEMIS- PHERE (HEM)	EXPERIMENTAL GROUP (EXP)			ANOVA
		RECALL (1)	AMNESIC (2)	WATER (3)	
DD	LEFT	0.877 (± 0.023)	0.884 (± 0.034)	0.916 (± 0.026)	EXP: F(2,18)=0.67, p=0.53
	RIGHT	0.790 (± 0.060)	0.872 (± 0.053)	0.834 (± 0.045)	HEM: F(1,18)=3.42, p=0.08
SL	LEFT	1.409 (± 0.026)	1.379 (± 0.030)	1.386 (± 0.034)	EXP: F(2,18)=0.07, p=0.93
	RIGHT	1.367 (± 0.015)	1.377 (± 0.019)	1.371 (± 0.019)	HEM: F(1,18)=1.94, p=0.18
STL	LEFT	0.721 (± 0.040)	0.717 (± 0.034)	0.723 (± 0.045)	EXP: F(2,18)=0.01, p=0.99
	RIGHT	0.688 (± 0.026)	0.699 (± 0.042)	0.701 (± 0.026)	HEM: F(1,18)=3.51, p=0.08
SHD	LEFT	0.688 (± 0.020)	0.663 (± 0.015)	0.663 (± 0.019)	EXP: F(2,18)=0.14, p=0.87
	RIGHT	0.674 (± 0.020)	0.678 (± 0.026)	0.668 (± 0.023)	HEM: F(1,18)=0.10, p=0.75
DL	LEFT	31.61 (± 2.630)	32.78 (± 2.895)	28.37 (± 1.886)	EXP: F(2,18)=0.47, p=0.63
	RIGHT	33.69 (± 2.210)	30.92 (± 1.882)	33.46 (± 2.691)	HEM: F(1,18)=0.56, p=0.46

Table 7: Mean values (in μm , \pm standard errors of the mean in brackets) of each of five dendrite and spine parameters examined for the three experimental groups (recall, amnesic and water). Corresponding ANOVA results are also given for the hemisphere (HEM) and experimental (EXP) effects. The interaction term is highly non - significant for all of the parameters examined and so is not included in this table. Number of chicks in each group = 7. DD=dendrite diameter, SL=spine length, STL=spine stem length, SHD=spine head diameter, and DL=dendrite length.

HEM	ORDER	TRAINED	CONTROL
LEFT	2	2.1591 (0.273)	2.0704 (0.206)
	3	3.0178 (0.788)	2.5825 (0.289)
	4	2.4375 (1.183)	1.8810 (0.555)
	5	1.1743 (0.983)	0.8721 (0.376)
	6	0.3030 (0.468)	0.1789 (0.164)
	7	0.0279 (0.079)	0.0416 (0.118)
RIGHT	2	1.9650 (0.108)	2.0423 (0.182)
	3	2.5816 (0.696)	2.4753 (0.656)
	4	1.8090 (1.095)	1.8911 (0.843)
	5	1.0592 (0.648)	0.7182 (0.640)
	6	0.2244 (0.213)	0.1320 (0.172)
	7	0.0350 (0.065)	0.0166 (0.047)

ANOVA SUMMARY		
EXPERIMENTAL (EXP):	$F(1, 14) = 1.06,$	n.s.
HEMISPHERE (HEM):	$F(1, 14) = 1.08,$	n.s.
EXP BY HEM:	$F(1, 14) = 0.40,$	n.s.
ORDER:	$F(1, 14) = 4567,$	$p < 0.0001$
EXP BY ORDER:	$F(1, 14) = 0.00,$	n.s.

TABLE 8: Number of branches of each order of dendrite per tree for LMPNs in the left and right hemispheres of trained and control chicks. Values in brackets are standard deviations. The results of ANOVA are also presented.

TABLE 9: Data for the ring intersection analysis. The values represent mean number of intersections per ring per dendritic tree for each neuron per chick (\pm SEMs in brackets) for 8 trained and 8 control chicks. Results of the ANOVA are given below (see text page.97).

HEM	RING	TRAINED	CONTROL
RIGHT	1	1.2263 (± 0.165)	1.4025 (± 0.082)
	2	2.7875 (± 0.162)	1.8038 (± 0.207)
	3	3.6800 (± 0.338)	3.2725 (± 0.286)
	4	3.9588 (± 0.391)	3.5963 (± 0.309)
	5	3.3325 (± 0.371)	3.1088 (± 0.390)
	6	2.5325 (± 0.353)	2.3338 (± 0.373)
	7	1.6663 (± 0.238)	1.4850 (± 0.364)
	8	0.7213 (± 0.133)	0.8575 (± 0.322)
	9	0.3775 (± 0.093)	0.4338 (± 0.166)
	10	0.1163 (± 0.039)	0.2313 (± 0.131)
	11	0.0063 (± 0.006)	0.0975 (± 0.056)
LEFT	1	1.3538 (± 0.097)	1.2375 (± 0.064)
	2	2.8775 (± 0.261)	2.6750 (± 0.190)
	3	3.9750 (± 0.414)	3.6375 (± 0.257)
	4	4.4662 (± 0.562)	3.7888 (± 0.193)
	5	4.1338 (± 0.571)	3.3900 (± 0.189)
	6	3.4750 (± 0.580)	2.7788 (± 0.177)
	7	2.1263 (± 0.401)	1.8013 (± 0.179)
	8	1.3400 (± 0.301)	1.0438 (± 0.161)
	9	0.7188 (± 0.190)	0.3775 (± 0.111)
	10	0.3188 (± 0.090)	0.0987 (± 0.040)
	11	0.1413 (± 0.047)	0.0138 (± 0.014)

ANOVA SUMMARY RESULTS			
EFFECT	RINGS 1 TO 4		RINGS 5 TO 8
EXP	F (1,14) = 0.97,	n.s	F (1,14) = 0.75, n.s
HEM	F (1,14) = 0.77,	n.s	F (1,14) = 3.67, n.s
EXP BY HEM	F (1,14) = 0.27,	n.s	F (1,14) = 0.57, n.s
RING	F (3,42) = 102.7,	p < 0.0001	F (3,42) = 148.3, p < 0.0001
EXP BY RING	F (3,42) = 1.16,	n.s	F (3,42) = 1.07, n.s
RING WITHIN HEM	F (6,84) = 260.8,	p < 0.0001	F (6,84) = 102.8, p < 0.0001
EXP BY RING WITHIN HEM	F (6,84) = 0.76,	n.s	F (6,84) = 0.72, n.s
EFFECT	RINGS 9 TO 11		
EXP	F (1,14) = 0.63,	n.s	
HEM	F (1,14) = 0.80,	n.s	
EXP BY HEM	F (1,14) = 4.41,	p < 0.06	
RING	F (2,28) = 32.07,	p < 0.0001	
EXP BY RING	F (2,28) = 0.74,	n.s	
RING WITHIN HEM	F (4,56) = 15.37,	p < 0.0001	
EXP BY RING WITHIN HEM	F (4,56) = 1.97,	n.s	

			GROUP	CORR. COEFF.	SIG.
VSD	x	SL	RECALL	+0.114	n.s.
			AMNESIC	-0.102	n.s.
			WATER	+0.08	n.s.
VSD	x	DD	RECALL	+0.198	n.s.
			AMNESIC	+0.449	p<0.001
			WATER	-0.061	n.s.
DD	x	SL	RECALL	-0.074	n.s.
			AMNESIC	-0.218	n.s.
			WATER	+0.091	n.s.

TABLE 10. Spearman correlations showing the effects of spine length (SL) and dendrite diameter (DD) on the visible spine counts (VSD) taken from the 2nd amnesia experiment. The significant positive correlation between VSD and DD for the recall group is unexpected: the thicker the dendrite, the more spines that are visible. However, there are no obvious correlations between the VSD and SL, suggesting at least that the increase in spine density after training is not mainly due to an increase in spine length (see text).

	LEFT TRAINED		LEFT CONTROL		RIGHT TRAINED		RIGHT CONTROL	
	F	F/N	F	F/N	F	F/N	F	F/N
Va	298	10.64	236	9.08	218	9.08	215	8.6
Vb	170	6.3	134	5.15	150	6.25	151	6.04
Vc	120	4.28	88	3.38	70	2.92	67	2.68
V-ta	12	0.43	13	0.5	11	0.458	9	0.36
V-tb	14	0.5	17	0.65	14	0.583	10	0.4
V-tc	7	0.26	12	0.462	10	0.417	8	0.32
V-td	3	0.11	7	0.27	3	0.125	5	0.2
V-1	9	0.33	10	0.38	11	0.458	13	0.52
V-4	6	0.22	2	0.08	3	0.125	4	0.16
V-5	4	0.148	0	0.	1	0.042	1	0.04

	GROUP	CHI-SQUARE	SIGNIFICANCE
Vd	TRAINING: LEFT	0.235	n.s.
	TRAINING: RIGHT	0.06	n.s.
	HEM. ASY: TRAINED	4.98	n.s.
	HEM. ASY: CONTROL	4.13	n.s.
V-t	TRAINING: LEFT	0.57	n.s.
	TRAINING: RIGHT	1.08	n.s.
	HEM. ASY: TRAINED	0.51	n.s.
	HEM. ASY: CONTROL	0.11	n.s.

TABLE 11. Topological data performed on dendritic trees of 52 LMPNs from trained and 51 from control chicks. The total frequencies of the different types of vertices (dichotomous: Va, Vb, Vc; trichotomous, V-ta, V-tb, V-tc, V-td; single, V-1; quadchotomous, V-4 and quintchotomous, V-5) are given in the left column under "F" and the frequency per neuron is given under "F/N", top table. The bottom table gives the chi-square analysis on the frequency values, (F) for the dichotomous (Vd) and trichotomous (V-t) vertices. No significant differences were found. Values for N are: left trained = 28; left control = 26; right trained = 24; and right control = 25. LEFT = left hemisphere; RIGHT = right hemisphere. HEM. ASY.=hemispheric asymmetry.

APPENDIX B

DERIVATION OF THE SPINE DENSITY CORRECTION FORMULA

The true total number of spines is given by the ratio:

$$N = \frac{n A_s}{A_c}$$

The formulae for A_s and A_c are derived geometrically from fig 7. The area A_s which is the area around the dendrite shaft containing all of the spines, not counting the areas occupied by the dendrite shaft and the surrounding zone of wall thickness SHD. The total area shown is $\pi(Dr + SL)^2$ and therefore

$$A_s = \pi [(Dr + SL)^2 - (Dr + SHD)^2]$$

The area A_c is the sum of the two visible flanking zones $A_c(1)$ and $A_c(2)$. Since spines whose lengths were less than the average diameter of spine heads were not counted, the area A_c represents that which is shaded in the diagram on the right of FIG. 9. $A_c(1) = A_c(2)$. $A_c(1)$ can be calculated by subtracting the area of the triangle MNO from the area of the sector of the circle bounded by lines MO and NO. The area of the sector is obtained by using t , which is 2θ , and t is given the cosine of θ :

$$\cos \theta = \frac{OP}{OM} = \frac{Dr + SHD}{(Dr + SL)}$$

The area of the sector MON will then be

$$\text{Area of sector MON} = \frac{t}{360} \pi (Dr + SL)^2$$

The area of the triangle MNO = 0.5 x MN.OP or MP.OP. The side OP can be determined by reference to the sine of θ :

$$MP = (Dr + SL) \sin \theta.$$

Thus the area of the visible flanking zones (Ac) will be given by $2 \times Ac(1)$, i.e.

$$Ac = 2 Ac(1) = 2 \left[\frac{t}{360} \pi (Dr + SL)^2 \right] - 2 (MP.OP)$$

Since $N = nAs/Ac$, therefore

$$N = \frac{n\pi[(Dr + SL)^2 - (Dr + SHD)^2]}{2 \left[\frac{t}{360} \pi (Dr + SL)^2 \right] - 2 (MP.OP)}$$

Substituting for t , MP , and OP and simplifying, the final formula becomes,

$$N = \frac{n\pi[(Dr + SL)^2 - (Dr + SHD)^2]}{2 \left[\frac{\theta}{90} \pi (Dr + SL)^2 \right] - 2 (Dr + SL) (Dr + SHD) \sin \theta}$$

APPENDIX C

THE RAPID GOLGI PROCEDURE:

CHEMICALS

FIXATIVE: 1% Paraformaldehyde, (mixed at 65-70°C, with a few drops of 1N NaOH).

1% Glutaraldehyde,

0.003% CaCl_2 ,

0.12M $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH=7.3) buffer.

WASHING MEDIUM: 8% Glucose,
0.003% CaCl_2 ,
0.12M $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH=7.3) buffer.

DURCUPAN (for gold-toning): 10g component A
10g component B
0.3-0.4g component C
0.3g component D.

PERFUSION: The chicks were anaesthetised with an overdose of sodium pentobarbitone. Once the chick failed to respond to a strong leg pinch, the dissection procedure was begun. The chicks' thoracic cavity was cut, exposing the heart. A fine needle, connected to a plastic (flexible tube) which passed through a perfusion pump (Watson-Marlow 502S), was inserted into the left ventricle and clamped in position. The right atrium was then punctured to allow blood to escape. Physiological saline (~15ml of 0.9%) was passed through at a high rate (9 ml/min) followed by ~120 ml of fixative. The characteristic erection of the chicks' fluff and feathers on the wings, together with a turning of the head and sometimes eye-opening normally marked the beginning of the fixation. The pump rate was then turned down (7 ml/min).

After 20-25 min of perfusion with the fixative, the brain was carefully removed and immersed in the same fixative for 24-48h. Using a specially prepared brain mould (Rose and Csillag, 1985), the left and right IMHV regions were dissected out and washed in washing medium for 2x15min. The blocks were then kept in 1% OsO_4 (dissolved in 0.12M $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH=7.3) buffer) for ~5h, after which the solution was changed, without washing, to 3.5% $\text{K}_2\text{Cr}_2\text{O}_7$ and left for a period of 48h. Following this, the $\text{K}_2\text{Cr}_2\text{O}_7$ was removed and the blocks were washed with 0.75% AgNO_3 but kept in fresh 0.75% AgNO_3 for 12-18h at 21°C in the dark. For sectioning, the blocks were embedded in 7% agar gel and cooled to 4°C before cutting with a Sorval tissue chopper into 90-120µm thick sections. The sections were temporarily mounted on slides with glycerol and the slides were subsequently coded.

At a later date (not more than 5 days of sectioning), the sections were dehydrated with 70% (5 min), 90% (5 min), 95% (5 min) and absolute (10-15 min) ethanol in that sequence, followed by histoclear. The sections were then layed on slides and permanently mounted in DPX. The edges of the coverslips were painted with nail varnish, to prevent the DPX from "shrinking".

THE GOLGI-GOLD TONING PROCEDURE (A modified Fairen et al (1977) procedure). For gold toning the Golgi-impregnated material is not permanently mounted in DPX. The tissue sections are examined under the light microscope while still mounted in glycerol, and the selected sections are exposed to bright, concentrated light for 20 min on both sides (by placing them on the light microscope stage and the illumination turned to near maximum - the exact setting is

determined by trial and error, and the type of microscope used). A heat filter (copper sulphate) has to be placed between the light source and the slide to protect the tissue. The gold-toning procedure is followed immediately. The sections to be gold toned are taken off the slide and:

---Incubated in ice cold gold solution (7 ml of 1% HAuCl_4 , $4\text{H}_2\text{O}$ and 2.5 ml glycerol in 100ml water), for 20min.

---Washed in ice - cold distilled water, 3x briefly.

---Incubated in ice cold 0.2% oxalic acid, for 2min.

---Washed in ice - cold distilled water, for 3x2 min.

---Washed in 1% sodium thiosulphate at $\sim 21^\circ\text{C}$, for 3x15 min.

---Washed in distilled water at $\sim 21^\circ\text{C}$ for 3x1-2 min.

This is followed by dehydration with 30% (5 min), 50% (10 min), 70% (15 min), 90% (10 min), 95% (10 min) and absolute (2x20 min) ethanol. The tissue is prepared for embedding in durcupan, by first leaving the sections in propylene oxide for 2x10 min and then in a 50:50 mixture of propylene oxide and durcupan for 12-18h at $\sim 21^\circ\text{C}$. The solution is changed to 100% durcupan and left for $\sim 12\text{h}$ (at $\sim 21^\circ\text{C}$) before mounting onto slides with 100% durcupan at 56°C , for light microscopy. The slides are first coated with silicone by rubbing them with a silicone impregnated lens tissue. This enables us to remove the section and durcupan from the slides, with relative ease, for electron microscopy.

For subsequent electron microscopy the section and a thin layer of durcupan are separated from the slide and the coverslip. The excess resin around the tissue section is cut away, and the flat piece of dried durcupan with the section, is placed on some durcupan that is already poured into an embedding mould (normally used

for producing resin blocks for electron microscopy), and covered with more resin. It is then left to dry at 56°C. The block is trimmed and prepared for ultrathin sectioning. The Golgi-gold toned cell can be cut in cross-section so that it is possible to follow a dendrite through the serial sections. Alternatively, the neuron can be serially sectioned *en face*, as seen when viewed under the light microscope. The sections are collected on formvar or pioloform coated slot grids. Photographs are then taken of the sections, initially at low magnification in order to identify gold toned dendrite shafts and follow them through adjacent sections. Higher magnification photographs are taken of the cell for quantitative studies of synapses and spines.